05-03-06

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re the Application of:	Group Art Unit: 1648
BLONDER et al.	Examiner: Li, Bao Q.
Serial No.: 09/888,235	
Conf. No.: 8106	SUBMISSION OF APPEAL BRIEF (37 C.F.R. § 41.37)
Filed: June 22, 2001	
Atty. File No.: 42830-00234	
For: "DELIVERY VEHICLE COMPOSITION AND METHODS FOR DELIVERING ANTIGENS AND OTHER DRUGS"	EXPRESS MAIL LABEL NO. EV738038835US

Mail Stop Appeal Brief – Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Submitted herewith is an Appeal Brief in relation to an appeal to an Office Action dated June 27, 2005. A Notice Of Appeal was previously filed on November 1, 2005. The appealed Office Action issued while the application was already on appeal and after the filing of a notice of appeal and an appeal brief relating to appealed rejections in that prior final Office Action. In a telephone conversation with Mr. Fred A Silverberg of the Office of Patent Legal Administration, in this situation, no fee under 37 C.F.R. § 41.20(b)(2) is due with filing of this Appeal Brief. This is because that fee was already been paid with previously-submitted appeal brief, which fee covers the filing of this Appeal Brief. Therefore, it is believed that no fees under

37 C.F.R. § 41.20(b)(2) are due in association with filing the enclosed Appeal Brief. However, if any such fees are due in association with filing the enclosed Appeal Brief, please debit such fees to Deposit Account No. 50-1419.

On April 28, 2006, a Petition For Extension of time under 37 C.F.R. § 1.136(a) to extend the time for filing this Appeal Brief for four months was filed along with a check for the required extension fee as provided in 37 C.F.R. § 1.17 for a four month extension, based on small entity status. Therefore, no additional extension should be required at this time for filing of the Appeal Brief. Also, no fees are believed to be due with this communication. If, however, any fees are due with this communication, please debit such fees to Deposit Account No. 50-1419. Also, if filing of this communication requires an additional available extension, please consider this document as including a petition for such extension and debit any required fees to Deposit Account No. 50-1419.

Respectfully submitted,

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Dear Sir:

This Appeal Brief is filed in relation to appeal of the referenced Application from claim rejections stated in an Office Action dated June 27, 2005 (hereinafter, "Appealed Office Action").

This Appeal Brief includes the following appendices:

Appendix A – Claims;

Appendix B – Evidence; and

Appendix C – Related Proceedings.

REAL PARTY IN INTEREST

The real party in interest is RxKinetix, Inc, a Delaware corporation, assignee of record of the Application.

RELATED APPEALS AND INTERFERENCES

None.

STATUS OF CLAIMS

Claims 1, 4-7, 9-31, 33-37, 39-44, 148, 149 and 150-197 are pending in the application, Claims 2, 3, 8, 32, 38 and 45-147 having been cancelled. Claims 1, 4-7, 9-31, 33-37, 39-44, 148 and 149 are the subject of this appeal. Claims 150-197 are withdrawn from consideration, as further discussed below.

Pending Claims 1, 4-7, 9-31, 33-37, 39-44, 148 and 149 are rejected under 35 U.S.C. § 1.02(b) or, in the alternative, under 35 U.S.C. § 103(a). It is noted that Claim 32 is encompassed within the claims stated as being pending on the Office Action Summary of the Appealed Office Action and as being rejected under 35 U.S.C. § 102(b) or, in the alternative, under 35 U.S.C. § 103(a), although it is believed that inclusion of Claim 32 was in error since that claim was previously cancelled.

Claims 1, 31, 33-44, 148 and 149 are rejected under 35 U.S.C. § 112, first paragraph, based on asserted lack of an enabling disclosure.

Claims 150-197 are withdrawn from consideration, and through an inadvertence during prosecution, Claims 150-197 are technically still pending in the application, even though those

claims are not listed in the Appealed Office Action. During prosecution, these claims were inadvertently dropped from the claim listing. There are no issues to address on appeal with respect to Claims 150-197, and an amendment has been submitted requesting cancellation of those claims, as discussed in the next section below.

STATUS OF AMENDMENTS

Two amendments of the claims have been filed that are not reflected in the Claims as presented in Appendix A.

1. An amendment was filed pursuant to 37 C.F.R. §§ 41.33 &1.116 on June 20, 2005 requesting cancellation of Claims 150-197. As of the time of filing this Appeal Brief, there has been no indication that the amendment has been entered or has been refused entry.
2. An amendment was filed pursuant to 37 C.F.R. §§ 41.33 &1.116 on April 28, 2006 to correct an obvious error in the preamble of Claims 148 and 149. As of the time of filing this Appeal Brief, there has been no indication that the amendment has been entered or has been refused entry.

SUMMARY OF CLAIMED SUBJECT MATTER

The claims subject to appeal are directed to a composition for delivery of an antigen (Claims 1, 4-7, 9-31, 33-37, 39-43, 148 and 149) and to a method of packaging and storing such a composition (Claim 44). (*See, inter alia*, specification generally at page 6, line 27, through page 9, line 19 and at page 10, lines 26-28.)

Claim 1, the only independent claim at issue in this appeal, is directed to a composition for delivery of an antigen, and recites the following components and features:

0.0001 weight percent to 5 weight percent of an antigen (*see, inter alia*, specification at page 15, lines 13-22 and page 20, lines 25-29);

5 weight percent to 33 weight percent polyoxyalkylene block copolymer that is biocompatible (*see, inter alia*, specification at page 17, line 23, through page 19, line 9 and original Claim 8);

60 weight percent to 85 weight percent aqueous liquid (*see, inter alia*, specification at page 20, lines 25-29);

0.01 weight percent to 10.0 weight percent additive, that is an adjuvant other than alum (*see, inter alia*, specification at page 7, lines 15-17, at page 20, lines 15-29 and original Claim 59); and

formulated with relative proportions of the liquid and the copolymer so that the copolymer interacts with the liquid to impart reverse thermal viscosity behavior to the composition, so that the viscosity of the composition increases when the temperature of the composition increases over some temperature range within 1°C and 37°C (*see, inter alia*, specification at page 16, lines 12-24 and at page, lines and original Claim 3).

Dependent Claims 4-7 each requires that the composition have properties with respect to gelation, with the composition being in the form of a flowable medium at a first, lower temperature, and in the form of a gel at a second, higher temperature. (*See, inter alia*, specification at page 13, line 27 through page 14, line 8 and original Claim 4.) Claims 5-7 each recite additional specificity with respect to gelation, summarized as follows:

Claim 5: the first temperature is in a range of from 1°C to 20°C (*see, inter alia*, specification at page 16, lines 24-31 and original Claim 5);

Claim 6: the first temperature is in a range of from 1°C to 20°C and the second temperature is a range of from 25°C to 37°C (*see, inter alia*, specification at page 16, lines 24-31 and original Claim 6);

Claim 7: the copolymer is substantially all dissolved in the liquid at the first temperature, and at least a portion of the copolymer comes out of solution when the temperature is raised from the first temperature to the second temperature (*see, inter alia*, specification at page 8, lines 12-17, at page 14, lines 5-13 at page 24, lines 3-7 and original Claim 7).

Dependent Claims 9-14 each requires that the copolymer comprises at least one block each of a first polyoxyalkylene and a second polyoxyalkylene. (*See, inter alia*, specification at page 17, lines 23-26, and original Claim 9.) Claims 10-14 each recite additional specificity with respect to the copolymer, summarized as follows:

Claim 10: the first polyoxyalkylene is polyoxyethylene and the second polyoxyalkylene is polyoxypropylene (*see, inter alia*, specification at page 17, lines 8-13 at page 17, line 27 through page 18, line 13, and original Claim 10);

Claim 11: the requirements of Claim 10, wherein the copolymer has the formula $HO(C_2H_4O)_b(C_3H_6O)_a(C_2H_4O)_bH$, wherein a and each b are independently selected integers (*see, inter alia*, specification at page 18, lines 19-33 and original Claim 11);

Claim 12: the formula of Claim 11, wherein the $(C_2H_4O)_b$ blocks together comprise at least 70 weight of the copolymer (*see, inter alia*, specification at page 18, lines 19-33 and original Claim 12);

Claim 13: the formula of Claim 11, wherein a is between 15 and 80 and each b is independently between 50 and 150 (*see, inter alia*, specification at page 18, lines 19-33 and original Claim 13);

Claim 14: the copolymer has the formula $H(OCH_2CH_2)_b(OCH(CH_3)CH_2)_a(OCH_2CH_2)_bOH$, wherein a is 20 to 80 and each b is independently 15 to 60 (*see, inter alia*, specification at page 18, lines 19-33, and original Claim 14).

Dependent Claims 15-31 each more particularly specifies the antigen (*See, inter alia*, specification at page 14, line 14 through page 15, line 12 and original Claims 15-31), with the subject matter of each of Claims 15-31 summarized as follows:

Claim 15: the antigen is selected from the group consisting of bacteria, protozoa, fungus, hookworm, virus and combinations thereof;

Claim 16: the antigen is selected from the group consisting of tetanus toxoid, diphtheria toxoid, a non-pathogenic mutant of tetanus toxoid, a non-pathogenic mutant of diphtheria toxoid and combinations thereof;

Claim 17: the antigen is from Bordatella pertussis;

Claim 18: the antigen is from influenza virus;

Claim 19: the antigen is from M. tuberculosis;

Claim 20: the antigen immunizes against a childhood illness;

Claim 21: the antigen is from rotavirus;

Claim 22: the antigen is selected from the group consisting of a polysaccharide, a peptide mimetic of a polysaccharide, an antigen from Neisseria meningitiditis, an antigen from Streptococcus pneumoniae and combinations thereof;

Claim 23: the antigen is from Epstein-Barr virus;

Claim 24: the antigen is from Hepatitis C virus;

Claim 25: the antigen is from HIV;

Claim 26: the antigen comprises a molecule involved in a mammalian reproductive cycle;

Claim 27: the antigen is HCG;

Claim 28: the antigen is a tumor-specific antigen;

Claim 29: the antigen is from a blood-borne pathogen;

Claim 30: the antigen is a first antigen and the composition comprises a second antigen;

Claim 31: the limitations of Claim 30, and wherein the first antigen is selected from the group consisting of tetanus toxoid, a nonpathogenic mutant of tetanus toxoid and combinations thereof; and the second antigen is selected from the group consisting of diphtheria toxoid, a nonpathogenic mutant of diphtheria toxoid and combinations thereof.

Dependent Claims 33-37 each more particularly specifies the adjuvant (*see, inter alia*, specification at page 20, lines 7-14 and original claims 33-37), with the subject matter of each of Claims 33-37 summarized as follows:

Claim 33: the adjuvant comprises dimethyl dioctadecyl ammonium bromide (DDA);

Claim 34: the adjuvant comprises a CpG motif;

Claim 35: the adjuvant comprises a cytokine;

Claim 36: the adjuvant comprises chitosan material;

Claim 37: the adjuvant comprises N,O-carboxymethyl chitosan.

Dependent Claims 39-43 each requires the composition to be in a particular form or contained in a particular device. Dependent Claims 39 and 40 each requires that the composition is in the form of disperse droplets in a mist, with Claim 40 also requiring that the mist is produced by a nebulizer. Dependent Claims 41 and 42 each requires that the composition is contained within a nebulizer actuatable to produce a mist comprising dispersed droplets of the composition, with Claim 42 also requiring that the nebulizer is a nasal nebulizer. Dependent Claim 43 requires that the composition is contained within an injection device actuatable to administer the composition to the host by injection. (*See, inter alia*, specification at page 7, lines 8-14, at page 22, lines 20-23, at page 23, line 28 through page 24, line 2 and original Claims 39-43.)

Claim 44 is directed to a method of packaging and storing the composition of Claim 5, and requires placing the composition in a container when the composition is in the form of a flowable medium and, after the placing, raising the temperature of the composition in the container to convert the composition to the gel form for storage, and wherein the gel form in the

container can be converted back to the form of a flowable medium for administration to the host by lowering the temperature of the composition in the container. (*See, inter alia*, specification at page 24, lines 8-18 and original Claim 44.)

Claim 148 requires that substantially all of the copolymer is dissolved in the liquid at some temperature within the temperature range recited in Claim 1, and Claim 149 requires that substantially all of the copolymer and the antigen are dissolved in the liquid at some temperature within the temperature range recited in Claim 1. (*See, inter alia*, specification at page 7, lines 27-32, at page 10, lines 4-6, at page 14, lines 5-13 and at page 24, lines 3-5.)

Each of Claims 1, 4-7, 9-31, 33-37, 39-44, 148 and 149 is separately reviewable on appeal. None of the claims are to stand or fall together.

GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

The following grounds for rejection in the Final Office Action are to be reviewed in this appeal:

Ground 1: Rejection of Claims 1, 4-7, 9-31, 33-37, 39-44, 148 and 149 under 35 U.S.C. § 102(b) as anticipated by EP 0 860 166 A1 by Alonso et al. (hereinafter "*Alonso et al.*") in view of US 5,607,691 by Hale et al. (hereinafter "*Hale et al.*") and US 5,300,295 by Viegas et al. (hereinafter "*Viegas et al.*")

Ground 2: Rejection of Claims 1, 4-7, 9-31, 33-37, 39-44, 148 and 149 under 35 U.S.C. § 103(a) as obvious over *Alonso et al.* in view of *Hale et al.* and *Viegas et al.*

Ground 3: Rejection of Claims 1, 31, 33-44, 148 and 149 under 35 U.S.C. § 112, first paragraph, as lacking an enabling disclosure.

ARGUMENT

The appealed claims involve a specific, but versatile composition for delivery of an antigen, in which the antigen is formulated with a non-alum adjuvant in a reverse thermal viscosity formulation, and in which a polyoxyalkylene block copolymer and an aqueous liquid interact to impart the reverse thermal viscosity behavior to the composition. Several example formulations are discussed in the application specification, including in the Examples section provided on pages 25 to 33 of the specification. Additional example formulations are discussed in a Rule 132 Declaration of Claire M. Coeshott (hereinafter The "Coeshott Declaration") submitted during prosecution, a copy of which is included in Appendix B. The rejections of claims made in the Appealed Office Action are not proper, and it is respectfully requested that all the rejections be reversed.

I. Ground 1, 35 U.S.C. §102(b)

The rejection of Claims 1, 4-7, 9-31, 33-37, 39-44, 148 and 149 under 35 U.S.C. § 102(b) as anticipated by *Alonso et al.* in view of *Hale et al.* and *Viegas et al.* is not proper. A copy of each of *Alonso et al.*, *Hale et al.* and *Viegas et al.* is included in Appendix B.

The invention as recited in Claim 1, the only appealed independent claim, is directed to a composition for delivery of an antigen, with the composition requiring, as noted above, at least four specified components each in a specified compositional range and with particular reverse

thermal viscosity behavior, summarized as follows:

(1) antigen, 0.0001 to 5 weight percent;

(2) polyoxyalkylene block copolymer, 5 to 33 weight percent;

(3) additive that is an adjuvant other than alum, 0.01 to 10 weight percent; and

(4) aqueous liquid, 60 to 85 weight percent; and

(5) reverse thermal behavior viscosity of the composition increases when the temperature of the composition increases over some temperature range within 1°C and 37°C.

To anticipate the subject matter of a claim under 35 U.S.C. § 102(b), each and every element as set forth in the claim must be found, either expressly or inherently, described in a single prior art reference. *Verdegaal Bros. v. Union Oil Co. of Calif.*, 814 F.2d 628, 631, 2 U.S.P.Q.2d 1051 (Fed. Cir. 1987). Moreover, an additional reference may be used only to assist in the interpretation of the single anticipating reference, and not for incorporation of any specific teachings of the additional reference into the anticipating reference. *Studiengesellschaft Kohle v. Dart Ind.*, 726 F.2d 724, 726-727, 220 U.S.P.Q. 841 (Fed.Cir.1984). Therefore, to anticipate the invention of Claim 1 for purposes of 35 U.S.C. § 102(b), *Alonso et al.* must disclose a composition for delivery of an antigen that expressly or inherently contains each and every limitation recited in Claim 1 in the specific combination recited in Claim 1. For purposes of 35 U.S.C. § 102(b), *Viegas et al.* and *Hale et al.* are available only to assist in the interpretation of the meaning of the teachings of *Alonso et al.*, and are not available for any other purpose.

A. *Alonso et al.* disclose drug delivery nanoparticles and a manufacture process for making

those nanoparticle, and the different teachings of *Alonso et al.* concerning the purpose and properties of the nanoparticles and of the purpose and properties of a formation medium used in the manufacture process must be distinguished.

Alonso et al. disclose nanoparticles designed for delivery of bioactive macromolecules. *Alonso et al.* also disclose a process for manufacture of the drug delivery nanoparticles, which process uses a "formation medium" as a process medium in which the nanoparticles are manufactured.

With respect to the properties of the nanoparticles of *Alonso et al.*, the following excerpts are quoted from *Alonso et al.* (with the excepts numbered for easy reference):

(i) Application of nanoparticles based on hydrophilic polymers as pharmaceutical forms for the administration of bioactive molecules. [*Alonso et al.*, at page 2, lines 3-4.]

(ii) The major constituents of these nanoparticles are two hydrophilic polymers: chitosan, which has a positive charge, and poly(oxyethylene), which has a non-ionic character. The active ingredient, which may be also a major constituent of these nanoparticles, is an antigenic or therapeutic macromolecule (peptide, protein, oligonucleotide, RNA, DNA . . .). The electrical charge of these colloidal particles can vary, depending on the ratio of the two hydrophilic polymers, from a highly positive value to a near zero value. The size of the nanoparticles can be modulated as well, from few nanometers to a few microns, by adequately selecting the preparation conditions. [*Alonso et al.*, at page 2, lines 5-10.]

(iii) Chitosan is a natural cationic polymer produced by deacetylation of the

polysaccharide chitin which is obtained from crustacean shells. Chitosan is available in the market in a variety of forms (with different molecular weights and degrees of deacetylation and, also, in the form of chitosan base or chitosan salt: e.g., hydrochlorhydrate, glutamate, lactate). [*Alonso et al.*, at page 2, lines 11-13.]

(iv) Poly(oxyethylene) or poly(ethylene oxide) (PEO) is a synthetic non-ionic polymer. PEO and its block copolymers with poly(propylene oxide) (PPO) are available in the market with different molecular weights and various ratios of ethylene oxide to propylene oxide groups. These block copolymers, especially the one containing 80% ethylene oxide, have been extensively used in the preparation of parenteral colloidal drug carriers because of their lack of toxicity. [*Alonso et al.*, at page 2, lines 14-17.]

(v) The new pharmaceutical composition described in this patent, based on the association of bioactive macromolecules to hydrophilic nanoparticles, overcomes problems previously encountered in the formulation of macromolecules. As indicated before, the main ingredients of the nanoparticles are two hydrophilic polymers: chitosan or chitosan salts and PEO or the block copolymers of poly(oxyethylene)-poly(oxypropylene) (PEO-PPO). The presence of PEO or PEO-PPO is not a requisite for the formation of the nanoparticles; however, the incorporation of these polymers in the system makes it more versatile since they affect the physicochemical properties of the nanoparticles such as the particles's [sic] size and zeta potential, as well as their release behavior and increase their biocompatibility. The chitosan:PEO ratio can vary enormously, reaching a value of 1:50. [*Alonso et al.*, at page 3, lines 3-10.]

(vi) The pharmaceutical systems described here are characterized in that they have a size smaller than 1 μm (nanoparticles) and a great capacity for the association of bioactive macromolecules. [*Alonso et al.*, at page 3, lines 36-37.]

(vii) 1. Application of nanoparticles made of hydrophilic polymers as pharmaceutical forms for the administration of bioactive macromolecules, characterized in that their main ingredients are an aminopolyssaccharide [sic] [e.g., chitosan], a salt from the phosphate family, a polyoxylethylene [sic] derivative [e.g., PEO-PPO] and an [sic] a high molecular weight bioactive ingredient. [*Alonso et al.*, claim 1, on page 7.]

(viii) 6. Pharmaceutical compositions, according to claim 1, characterized in that said poly(oxylethylene) [sic] derivative is selected from the group of the poly(oxylethylene) [sic] and the block copolymers of the ethylene oxide and propylene oxide [PEO-PPO]. [*Alonso et al.*, claim 6, on page 7.]

(ix) 10. Pharmaceutical compositions, according to claim 6, characterized in that said poly(oxylethylene) [sic] derivative [e.g., PEO-PPO polymer] is incorporated in a percentage with respect to the total weight of the composition comprised between 0 % and 60 % (w/w). [*Alonso et al.*, claim 10, on page 8.]

With respect to the manufacture process disclosed by *Alonso et al.* for making the nanoparticles, and the formation medium used in that manufacture process, the following excerpts are quoted from *Alonso et al.* (with the excepts numbered for easy reference):

(x) The incorporation of bioactive macromolecules within the nanoparticles can be achieved by a very simple and mild procedure which is particularly effective for preserving the stability of the macromolecules. [*Alonso et al.*, at page 2, lines 21-22.]

(xi) The formation of the nanoparticles occurs spontaneously due to the simultaneous

precipitation of chitosan and the bioactive macromolecule caused by the incorporation of a molecule with a basic character, i.e., sodium tripolyphosphate (counter anion). This process can be also considered as a process of ionic gelation or ionic crosslinking of chitosan with the counter anion. In this method, the utilization of organic solvents, extreme pH conditions or auxiliary substances of toxic nature are avoided. [*Alonso et al.*, at page 2, lines 23-27.]

(xii) The association of bioactive macromolecules with the nanoparticles occurs by a combined mechanism which may involve ionic and non-ionic interactions between the bioactive macromolecule and chitosan and a physical entrapment process. The ionic interaction between chitosan and negatively charged polymers has been previously described as the main mechanism involved in the formation of microcapsules by complex coacervation [citation omitted] and of polyion complexes [citation omitted]. However, the association of bioactive macromolecules to nanoparticles made of chitosan or chitosan-PEO, according to an ionic interaction mechanism, has not yet been described. In addition, the originality here relies in the fact that the incorporation of the bioactive macromolecule into the nanoparticles occurs upon the incorporation of an ionic crosslinking agent such as sodium tripolyphosphate. [*Alonso et al.*, at page 2, lines 28-38.]

(xiii) The size of the nanoparticles is mainly dependent on the chitosan concentration in the nanoparticles formation medium. Thus, for a very low chitosan aqueous concentration (lower than 0.01%) or a very high chitosan aqueous concentration (higher than 0.5%), an aqueous gel solution or a suspension of microparticles (larger than 1 μm) is formed respectively. In addition, the size of the particles can be also modulated by incorporating PEO or PEO-PPO in the nanoparticles formation medium. As an example, results presented in Table 1 show the important augmentation in the nanoparticle size (from 275 nm to 685 nm) caused

by the incorporation of increasing amounts of PEO-PPE [sic]in the medium (the chitosan/PEO-PPO ratio varied from 1/10 up to 1/50). Results in Table 1 also show that the incorporation of PEO-PPO to the nanoparticles led to significant reduction in their zeta potential values. [*Alonso et al.*, at page 3, lines 37-45.]

(xiv) 2. Pharmaceutical compositions, according to claim 1, characterized in that they are formed spontaneously in an aqueous medium, by ionic crosslinking and subsequent precipitation of the aminopolyssaccharide [sic] [e.g., chitosan]. [*Alonso et al.*, claim 2, on page 7.]

B. The Examiner's characterization of *Alonso et al.* confuses teachings by *Alonso et al.* concerning the purpose and properties of the drug delivery nanoparticles of *Alonso et al.* and the purpose and properties of the formation medium used in the manufacture process by which those drug delivery nanoparticles are made.

On page 2 of the Appealed Office Action, in paragraph 3, the Examiner states:

Alonso et al. teach a method of formulating an immunogenic composition comprising an antigen with an adjuvant chitosan and a polyoxyalkylene block copolymer in a liquid form, wherein the polyoxyalkylene block copolymer is PEO-POP [sic], which is the same copolymer as it is taught in the current specification . . . *Alonso et al.* also disclose that the proportions of each active ingredient varied according to the size of the nanoparticle, some of the changes are within the claimed ranges. For example, the composition having the nanoparticle with the size of $685^{\pm}27$ comprising about 0.14% (w/w) of chitosan , 0.014% (w/w) of tetanus toxoind [sic], 0.02% (w/w) sodium triphosphate [sic], 7% (w/w) of PEO-PPO and 93% (w/w) water. (*See*, Table 1 on page 6 and Example 4 on page 5.) Moreover, *Alonso et al.* teach that...the total weight of the copolymer may vary from 0%

to 60% (Claims 6-13).

The specific combination of constituents asserted by the Examiner to be disclosed by *Alonso et al.* cannot be found in *Alonso et al.*, either expressly or inherently, and, as discussed in detail below, neither the nanoparticles nor the formation medium disclosed by *Alonso et al.* anticipate the subject matter of the appealed claims. The compositional combinations purported by the Examiner to be disclosed by *Alonso et al.* appear to combine out of context a number of different teachings of *Alonso et al.* concerning the nanoparticles and formation media used to make the nanoparticles.

C. The drug delivery nanoparticles disclosed by *Alonso et al.* do not contain the combination of components recited in appealed Claim 1.

From excerpts (ii), (v) and (vii) above, *Alonso et al.* disclose that the "major constituents" or "main ingredients" of the nanoparticles of *Alonso et al.* are two hydrophilic polymers, chitosan and PEO or PEO-PPO, and also possibly the macromolecular active ingredient that is to be delivered by the nanoparticles. *Alonso et al.* make only very limited disclosures concerning the compositional ranges in the nanoparticles for these "major constituents". In except (v) above, *Alonso et al.* disclose that the chitosan:PEO ratio in the nanoparticles "can vary enormously, reaching a value of 1:50," and in excerpt (ix) above *Alonso et al.* disclose that the PEO or PEO-PPO may be "incorporated in a percentage with respect to the total weight of the composition comprised between 0 % and 60 % (w/w)."

There is no disclosure in *Alonso et al.* that the nanoparticles of *Alonso et al.* comprise any significant amount of an aqueous liquid. The only disclosures in *Alonso et al.* concerning an aqueous liquid is with respect to the formation medium disclosed by *Alonso et al.* for use to make the nanoparticles by precipitation. *See*, excerpts (xiii) and (xiv) above and Examples 1-5 on pages 4-6 of *Alonso et al.* In asserting that *Alonso et al.* disclose a composition that may include both PEO-PPO in a range of 0-60% and a high water content within the scope of the appealed Claims, the Examiner has apparently confused disclosures by *Alonso et al.* concerning the make-up of the nanoparticles and the make-up of the aqueous formation medium used to make the nanoparticles. The nanoparticles disclosed by *Alonso et al.* contain as "major constituents" only chitosan, PEO or PEO-PPO, and possibly a macromolecular active ingredient, which means that water is necessarily excluded as a component making up any significant part of the nanoparticles of *Alonso et al.* In contrast, the composition recited in appealed Claim 1 requires from 60 weight percent to 85 weight percent of an aqueous liquid. The nanoparticles disclosed by *Alonso et al.* do not contain an aqueous liquid in the claimed range.

D. The formation medium disclosed by *Alonso et al.* does not contain the combination of components recited in appealed Claim 1.

It is noted that it is the nanoparticles that *Alonso et al.* discloses as being designed for delivery of macromolecular active ingredients, and not the formation medium. The formation medium of *Alonso et al.* is a process medium used to make the nanoparticles. Nevertheless, the formation medium of *Alonso et al.* still does not contain the combination of components of the

composition for delivery of an antigen recited in appealed Claim 1.

1. *Alonso et al.* do not expressly disclose formation medium for making nanoparticles that contains from 60 weight percent to 85 weight percent aqueous liquid.

The only disclosures by *Alonso et al.* showing water content in the formation medium used to make nanoparticles is in Examples 1-5, presented on pages 4-6 of *Alonso et al.* For each of these Examples, *Alonso et al.* provide a table showing the composition of the formation medium used for the Example and listing the content of water as being "up to 100%," so that water makes up the balance of each formation medium after accounting for the other listed ingredients. By simple calculation, the water content, on a weight percentage basis, in the formation medium of each of the Examples of *Alonso et al.* is: 99.826% water in Example 1; 99.126% water in Example 2; 96.326% water in Example 3; 99.826% water in Example 4; and 99.833% water in Example 5. Therefore, the lowest water content disclosed by *Alonso et al.* in formation medium used to make nanoparticles is 96.326% by weight, which is outside of the range of from 60 to 85 weight percent aqueous liquid recited in appealed Claim 1. *Alonso et al.* do not expressly disclose any formation medium containing the Examiner's asserted 93% water. As discussed below, a 93% water content in a formation medium is also not inherently disclosed by *Alonso et al.*, but even if such a water content were inherently disclosed, such a water content would still be outside of the range of from 60 to 85 weight percent aqueous liquid recited in appealed Claim 1.

2. *Alonso et al.* do not expressly disclose formation medium for making nanoparticles that contains from 5 weight percent to 33 weight percent polyoxyalkylene block copolymer.

The only disclosures by *Alonso et al.* showing PEO-PPO content in formation medium used to make nanoparticles is in Examples 1-5, on pages 4-6 of *Alonso et al.* The largest PEO-PPO concentration in a formation medium disclosed by *Alonso et al.* in those Examples is 3.5% by weight (Example 3), which is outside of the range of from 5 to 33 weight percent polyoxyalkylene block copolymer recited in appealed Claim 1. *Alonso et al.* do not expressly disclose formation medium containing the Examiner's asserted 7% PEO-PPO. As discussed below, a 7% PEO-PPO content in the formation medium is also not inherently disclosed by *Alonso et al.*

The Examiner cites to the disclosure in claims 6-13 of *Alonso et al.* for the proposition that *Alonso et al.* disclose a composition containing between 0% and 60% polyoxyethylene-polyoxypropylene block copolymer, but a review of that portion of *Alonso et al.* reveals that the reference made by *Alonso et al.* to that concentration range must be in relation to the nanoparticles, and not the formation medium. The only place where *Alonso et al.* state the 0% to 60% range for polyoxyethylene-polyoxypropylene block copolymers is in claim 10 of *Alonso et al.* (*See*, excerpt (ix) above.) Claim 10 of *Alonso et al.* depends from claim 6, which in turn depends from claim 1. It is noted that the claims presented on pages 7 and 8 of *Alonso et al.* contain many internal textual inconsistencies. The preamble portion of claim 1 of *Alonso et al.*, the only independent claim, states that the subject matter of the claim is directed to "Application of nanoparticles" whereas the preamble portions of all dependent claims refer to "Pharmaceutical

compositions, according to [prior claim or claims]". Although the text in the preamble portions of the claims is not consistent, it is clear that the "Pharmaceutical compositions" in the dependent claims must be in reference to the "nanoparticles" stated in claim 1 of *Alonso et al.*, otherwise the claim set would make no sense. Therefore, the statement in Claim 10 of *Alonso et al.* that the 0% to 60% range for the "poly(oxylethylene) [sic] derivative" is "with respect to the total weight of the composition" must be in reference to the weight of the nanoparticles, and not in reference to the weight of a formation medium that may have been used in a manufacture process in which the nanoparticles were made.

3. *Alonso et al.* do not inherently disclose formation medium for making nanoparticles that contains either 93% water or 7% PEO-PPO as asserted by the Examiner.

In asserting in the Appealed Office Action that *Alonso et al.* disclose a composition with 7% PEO-PPO and 93% water, the Examiner specifically referred to Table 1 on page 6 of *Alonso et al.* and Example 4 on page 5 of *Alonso et al.* Example 4 of *Alonso et al.* concerns making nanoparticles from a formation medium containing tetanus toxoid as the active ingredient, but containing no PEO-PPO. Table 1 of *Alonso et al.* summarizes data for nanoparticles made using different chitosan/PEO-PPO ratios (from 1/0 to 1/50), but nowhere does *Alonso et al.* disclose the composition of the formation media used to generate the data for Table 1, so that the concentrations of PEO-PPO and water in formation media used to generate the data for Table 1 are not known.

Although not stated in the Appealed Office Action, it may be that the Examiner's position

that *Alonso et al.* disclose a composition containing 93% water and 7% PEO-PPO is based on an assumption that the concentration of chitosan in formation media used to generate the data of Table 1 is the same as the concentration of chitosan in the formation media disclosed by *Alonso et al.* in Examples 1-5, and that based on that assumption, the concentration of PEO-PPO in the formation media used to generate the data for Table 1 can be calculated using that chitosan concentration and the ratios of chitosan to PEO-PPO disclosed in Table 1. Examples 1-5 of *Alonso et al.* each used a formation medium containing 0.14% by weight chitosan. If it is assumed that the formation media used to generate the data of Table 1 of *Alonso et al.* also contained 0.14% by weight chitosan then by calculation, the formation medium used to make the nanoparticles presented in Table 1 for the 1/50 ratio of chitosan to PEO-PPO would contain PEO-PPO at a concentration of 7% by weight (50 x 0.14), and by difference the same formation medium would contain approximately 93% by weight water. But if the Examiner's position is based on such an assumption that the concentration of chitosan in the formation media used to generate the data of Table 1 of *Alonso et al.* is the same as used in the formation media of Examples 1-5 of *Alonso et al.*, that position is untenable.

Because *Alonso et al.* do not expressly disclose a formation medium containing 7% by weight PEO-PPO and approximately 93% water, the Examiner's assertion must be based on a position that the unexpressed concentrations for PEO-PPO and water for the formation media used to generate the data of Table 1 are inherently disclosed by *Alonso et al.*, using the assumption concerning chitsoan concentration as discussed above.

Just because it is possible that some combination or combinations of operating parameters might conceivably lead to production of a product with the claimed features, that does

not raise an issue of inherency with respect to the product. It is well settled that the allegedly inherent feature must necessarily result, and not just be possible or probable. *Transclean Corp. v. Bridgewood Services, Inc.*, 290 F.3d 1364, 1373, 62 U.S.P.Q.2d 1865 (Fed. Cir. 2002). The mere fact that a certain thing may result is not enough. *Mehl/Biophile International Corp. v. Milgraum*, 192 F.3d 1362, 1365, 52 U.S.P.Q.2d 1303 (Fed. Cir. 1999). Anticipation of product inventions cannot be predicated on mere conjecture respecting the characteristics of products that might result from a disclosed process. *W.L. Gore & Associates, Inc. v. Garlock, Inc.*, 721 F.2d 1540, 1554, 220 U.S.P.Q. 303 (Fed. Cir. 1983).

The case of *In re Best*, 562 F.2d 1252, 195 U.S.P.Q. 430 (CCPA 1977) is instructive. At issue in *Best* were both process claims for making a zeolitic material and product claims to the zeolitic material. The process claims required a heat treatment step followed by a cooling step, with a recited limitation that a rate of cooling during the cooling step must be sufficiently rapid that the cooled zeolitic material exhibits a certain X-ray powder diffraction pattern. The product claims required that the zeolitic material have certain compositional characteristics, including the same X-ray powder diffraction pattern used to define the cooling step of the process claims. During prosecution, claims to the process were rejected for lack of novelty and as being obvious based on a reference that expressly disclosed all positive process limitations except for the functionally expressed rate of cooling. The rejection of the process claims was premised on an assertion by the Examiner that there was no indication that the functionally expressed rate of cooling would in any way differ from a normal rate of cooling resulting from removal of the heat source following heat treatment during the prior art process of the cited reference. The Examiner then rejected the product claims on the basis that the claimed product was the unique result of the

claimed process, and that the same product would therefore result from the same process disclosed by the prior art reference. On appeal, the Court of Customs and Patent Appeals (CCPA) upheld the rejections. With respect to the process claims, the CCPA in *Best* stated:

> Our reading of Hansford [the cited prior art reference] leads us to conclude, as did the board, that all process limitations of claim 3 are expressly disclosed by Hansford, except for the functionally expressed rate of cooling. Because any sample of Hansford's calcined zeolitic catalyst would necessarily be cooled to facilitate subsequent handling, the conclusion of the examiner that such cooling is encompassed by the terms of the appealed claims was reasonable. [*Best,* 562 F.2d at 1255, emphasis added.]

In upholding the rejection of product claims, the CCPA in *Best* stated:

> Though urging that the other parameters are the unique result of their claimed process, appellants have offered no comparison of those other parameters with the corresponding parameters of Hansford's product. [*Best,* 562 F.2d at 1256.]

An important aspect of the *Best* decision is that the unexpressed cooling step in the prior art reference was inherently specified by a recognition that at least ambient cooling is necessarily implied to occur when heat treatment of the prior art reference is discontinued, and therefore all of the prior art process steps were specified to an extent permitting a comparison product to be prepared for comparison with the requirements of the product claims (e.g. the X-ray powder diffraction pattern of the resulting product).

As noted above, *Alonso et al.* do not disclose the composition of formation media used to

generate the data of Table 1, to which the Examiner made reference in asserting that *Alonso et al.* disclose a composition containing 7% by weight PEO-PPO and 93% by weight water. Although there may be some possibility that the formation media processed by *Alonso et al.* to generate the data of Table 1 contained chitosan at the same concentration as that used in the formation media of Examples 1-5 (0.14% by weight), a mere possibility is not enough to maintain a rejection based on inherency. It would have to be a necessary condition, and that is not the case here.

The only statement made by *Alonso et al.* concerning Table 1 is quoted in excerpt (xiii) above. Other than the relative ratios of chitosan to PEO-PPO tested, there is no information concerning the composition of the formation media. Moreover, the quote in excerpt (xiii) makes clear that *Alonso et al.* contemplate the use of chitosan concentrations ranging from as low as 0.01% to 0.5%, foreclosing a finding that the data of Table 1 must necessarily have been generated using formation media with a chitosan concentration of 0.14%. Moreover, it is noted that none of the nanoparticles reported in Table 1 of *Alonso et al.* have a size that is the same as any of the nanoparticles reported in any of Examples 1-5 of *Alonso et al.*, again foreclosing a finding that the data of Table 1 must necessarily have been generated using formation media with the same chitosan concentration as used in Examples 1-5.

There is nothing in *Alonso et al.* on which a *prima facie* rejection could be made based on an inherent disclosure by *Alonso et al.* of a composition, as asserted by the Examiner, that contains 7% by weight PEO-PPO or 93% by weight water.

4. *Alonso et al.* do not disclose, expressly or inherently, any compositions that have reverse thermal viscosity behavior, such that the viscosity of the composition increases over some

temperature range within 1°C to 37°C.

There is no express disclosure in *Alonso et al.* that the drug delivery nanoparticles of *Alonso et al.* or the formation medium used to make those nanoparticles have a reverse thermal viscosity property as recited in appealed Claim 1. The Examiner does not assert that *Alonso et al.* expressly disclose that any of their compositions exhibit reverse thermal viscosity behavior. The Examiner's position, although not stated in the Appealed Office Action, appears to be that the compositions disclosed by *Alonso et al.* that contain polyoxyethylene-polyoxypropylene block copolymer (abbreviated by *Alonso et al.* as "PEO-PPO", as noted above) would inherently have that characteristic, perhaps with reference to *Viegas et al.* for interpretative support. But the Examiner's reliance on inherency is misplaced.

Initially, it is noted that reverse thermal viscosity behavior is not a property of a polymer, but of a mixture of certain polymers with water. It is the interaction of the polymer and the water that results in the reverse thermal viscosity property. Not all polymers, including not all polyoxyalkylene block copolymers, can be formulated with water to produce reverse thermal viscosity behavior. Also, for those polymers that can be formulated with water to produce reverse thermal viscosity behavior, the reverse thermal viscosity behavior does not result for all possible proportions of the polymer and water.

As to the drug delivery nanoparticles of *Alonso et al.*, as discussed above, these nanoparticles do not contain a significant amount of water, which would be required for the nanoparticles to exhibit reverse thermal viscosity behavior. Also, for both of the drug delivery nanoparticles and the formation media of *Alonso et al.*, there is no disclosure by *Alonso et al.* as

to what particular PEO-PPO material is used by *Alonso et al.* to make the nanoparticles.

Alonso et al. do not disclose the particular PEO-PPO material used in the Examples presented by *Alonso et al.* or to generate any of the data presented in Tables 1-5 on pages 5 and 6 of *Alonso et al.* For example, *Alonso et al.* do not disclose the molecular weight of the PEO-PPO material used, or the relative percentages of PEO and PPO contained the PEO-PPO material used. Without that information, it is impossible to determine whether or not any of the compositions disclosed by *Alonso et al.* necessarily exhibit reverse thermal viscosity behavior. The disclosure of *Viegas et al.* provides no interpretative assistance as to whether the specific PEO-PPO used by *Alonso et al.* in the specific compositional formulations disclosed by *Alonso et al.* would or would not necessarily exhibit reverse thermal viscosity behavior.

The only disclosure made by *Alonso et al.* as to any particular PEO-PPO material is as quoted in excerpt (iv) above, concerning block copolymers containing 80% ethylene oxide that are disclosed as having been extensively used in the preparation of parenteral colloidal drug carriers because of their lack of toxicity. But *Alonso et al.* do not disclose that such block copolymers containing 80% ethylene oxide were the PEO-PPO materials actually used in any of the Examples presented by *Alonso et al.* or to generate any of the data for Tables 1-5 of *Alonso et al.* As discussed above, anticipation based on inherency requires more than a mere possibility that a particular PEO-PPO material may have been used by *Alonso et al.* It is possible that *Alonso et al.* used a PEO-PPO material containing 80% ethylene oxide for the Examples, but it is also possible that they did not.

Moreover, even if, solely for the sake of argument, the PEO-PPO used in the examples of *Alonso et al.* is assumed to contain 80% ethylene oxide, there is still no disclosure by *Alonso et*

al. as to the molecular weight of the PEO-PPO that was used, which information would be required before it could be determined whether any of the compositions disclosed by *Alonso et al.* necessarily exhibit reverse thermal viscosity behavior.

In that regard, reference is made to Newman, M. J. Balasubramanian M., and Todd C.W, "Development of adjuvant-active nonionic block copolymers," Advanced Drug Delivery Reviews 32 (1998), pp. 199-223 (hereafter referred to as "*Newman et al.*"), which is of record in the prosecution of this application, and a copy of which is included in Appendix B. Referring to pages 201-202 of *Newman et al.,* there is a discussion concerning conventions for naming polyoxyethylene-polyoxypropylene block copolymers based on molecular weight and weight percent polyoxyethylene content in the copolymer. Fig. 2 presented on page 202 of *Newman et al.* shows a grid plotting several different polyoxyethylene-polyoxypropylene block copolymers with respect to polymer molecular weight and percent polyoxyethylene content in the polymer. Notably, there are six different polymers plotted that contain 80% polyoxyethylene, each of a different molecular weight ranging from as small as 950 to as high as 3250, clearly demonstrating that there are several possibilities even for the 80% ethylene oxide block copolymers noted by *Alonso et al.*, and negating any possibility that *Alonso et al.* could inherently disclose any particular PEO-PPO material containing 80% polyethylene oxide.

Reference is also made to U.S. Patent No. 5,702,717 by Cha et al. (hereinafter referred to as "*Cha et al.*"), which is of record in the prosecution of this application, and a copy of which is included in Appendix B. *Cha et al.* provide a discussion, at column 3, line 51 through column 4, line 39, of the reverse thermal gelation properties of certain polyoxyethylene-polyoxypropylene block copolymers marketed under the Pluronic™ or Poloxamer™ tradenames. As discussed in

the present Application, reverse thermal gelation is one form of reverse thermal viscosity behavior. Notably, in Table 1, in column 4 of *Cha et al.*, data is presented by *Cha et al.* concerning the gel-forming behavior of five different polyoxyalkylene block copolymers at different concentrations. Four of the polyoxyalkylene block copolymers listed in Table 1 of *Cha et al.* are different molecular weight polymers that each contains 80% polyoxyethylene (F-68, F-88, F-98, and F-108) with three of these (F-68, F-98 and F-108) being polymers that were also shown on the grid disclosed in *Newman et al.*, discussed above. As shown in Table 1 of *Cha et al.*, these polymers of different molecular weight exhibit different gelling behavior in aqueous solution, with the F-68 polymer not exhibiting gelation until the concentration is increased to 40% and the temperature increased to 37°C. This demonstrated disparity in viscosity behavior among different molecular weights of polyoxethylene-polyoxypropylene block copolymers containing 80 weight percent polyoxyethylene further negates a possible finding that *Alonso et al.* discloses any compositions that necessarily exhibit reverse thermal viscosity behavior.

Referring again to the *Best* decision, discussed above, the situation with the present application in relation to reverse thermal viscosity behavior is different than the type of situation presented in *Best*, where inherency was found. In *Best*, the conditions of the prior art process to be used for making a product for comparison were specified for each processing step, either expressly or by necessary implication. With respect to the teachings of *Alonso et al.* in relation to the subject matter of Claim 1, the PEO-POP used by *Alonso et al.* in the formation media of the Examples of *Alonso et al.* is not expressly supplied or necessarily implied. For example, if the Applicant were to repeat the Examples of *Alonso et al.* to test for the presence of reverse-thermal viscosity behavior, what relative percentages of polyethylene oxide and polypropylene

oxide should the PEO-PPO material contain, and what molecular weight should the PEO-PPO material be? There is clearly no specification, by either express disclosure or necessary implication, of the processing conditions used by *Alonso et al.* (and especially concerning the specific PEO-PPO material used) that would form the basis of a test to determine whether those conditions necessarily result in a product including the reverse thermal viscosity limitations recited in Claim 1. Shifting of the burden of proof to require an applicant to disprove inherency of a product simply does not apply in this situation, because *Alonso et al.* does not provide sufficient disclosure to raise a *prima facie* case of inherency.

E. *Viegas et al.* and *Hale et al.* are available only to aid interpretation of the disclosures of *Alonso et al.*, and as interpretational aids do not alter the above discussion concerning deficiencies of *Alonso et al.*

As noted above, for purposes of 35 U.S.C. § 102(b), *Viegas et al.* and *Hale et al.* are available only to assist in the interpretation of the meaning of the teachings of *Alonso et al.* *Viegas et al.* concern compositions for ophthalmic drug delivery, and the Examiner cited to *Viegas et al.* specifically in relation to the disclosure by *Viegas et al.* of heat-sensitive gelation properties of certain formulations involving certain polymers disclosed by *Viegas et al.*, but the teachings of *Viegas et al.* do not alter the interpretations of the teachings of *Alonso et al.* discussed above. *Hale et al.* concern delivering pharmaceutical agents across membranes, including the skin layer or mucosal membranes, using a pharmaceutical agent covalently bonded to a chemical modifier via a physiologically cleavable bond. The Examiner cited to particular

portions of *Hale et al.* that relate to passive transdermal delivery across a membrane, which may be of a polyethylene material, and to nasal/pulmonary administration, which may involve employment of an aerosol formulation. The particular teachings of each of *Viegas et al.* and *Hale et al.* are discussed in detail below concerning Ground 2, but neither the teachings of *Viegas et al.* nor the teachings of *Hale et al.* alter the interpretations of the teachings of *Alonso et al.* discussed above.

G. Dependent claims recite one or more additional feature that, in combination with the limitations of Claim 1, further distinguish over *Alonso et al.*

With respect to appealed dependent Claims 4-7, 9-31, 33-37, 39-44, 148 and 149, these claims are also not anticipated by *Alonso et al.* for the same reasons as stated above with respect to independent Claim 1. Moreover, each of these dependent claims contains one or more limitations in addition to the limitations recited in appealed Claim 1, which additional limitation or limitations in combination with the limitations of Claim 1 further distinguish over *Alonso et al.*

Claims 4-7: Each of dependent Claims 4-7 requires a feature recited in Claim 4 that the claimed composition has properties with respect to gelation, with the composition being in the form of a flowable medium at a first, lower temperature, and in the form of a gel at a second, higher temperature. This reverse thermal gelation requirement is a particular type of reverse thermal viscosity behavior which is not expressly or inherently disclosed by *Alonso et al.* Moreover, Claim 5 requires that the first temperature recited in Claim 4 be in a range of 1°C to

20°C; Claim 6 requires that the first temperature recited in Claim 4 be in a range of from 1°C to 20°C and the second temperature recited in Claim 4 be in a range of from 25°C to 37°C; Claim 7 requires that the copolymer is substantially all dissolved in the liquid at the first temperature, and at least a portion of the copolymer comes out of solution when the temperature is raised from the first temperature to the second temperature. *Alonso et al.* simply do not disclose, expressly or inherently, any of these additional requirements concerning particular viscosity behavior of the claimed composition.

Claims 9-14: Each of dependent Claims 9-14 requires a feature recited in Claim 9 that the polyoxyalkylene block copolymer comprises at least one block each of a first polyoxyalkylene and a second polyoxyalkylene. Claim 10 is dependent from Claim 9, and further requires that the first polyoxyalkylene is polyoxyethylene and the second polyoxyalkylene is polyoxypropylene; Claim 11 is dependent from Claim 10 and further requires that that the polyoxyalkylene block copolymer has the formula $HO(C_2H_4O)_b(C_3H_6O)_a(C_2H_4O)_bH$, wherein a and each b are independently selected integers; Claim12 is dependent from Claim 11 and further requires that the $(C_2H_4O)_b$ blocks together comprise at least 70 weight of the copolymer; Claim 13 is dependent from Claim 11 and further requires that the "a" variable in the recited formula is between 15 and 80 and each "b" variable in the recited formula is independently between 50 and 150; and Claim 14 is dependent from claim 10 and further requires that the polyoxyalkylene block copolymer has the formula $H(OCH_2CH_2)_b(OCHCH_2)_a(OCH_2CH_2)_bOH$ (with CH_3 on the $OCHCH_2$ group), wherein the "a" variable is 20 to 80 and each "b" variable is independently 15 to 60. As discussed above, *Alonso et al.* do mention polyoxyethylene-polyoxypropylene block copolymers "containing 80% ethylene oxide",

although not in the claimed combination, but *Alonso et al.* do not at all disclose polyoxyalkylene block copolymers comprising two polyoxyethylene blocks and one polyoxypropylene block as required of each of Claims 11-14 or any of the particular formulae required of any of Claim 11, Claim 12, Claim 13 or Claim 14.

Claims 15 and 17-31: Each of dependent Claims 15 and 17-31 requires inclusion of particular antigen material in the claimed composition, with Claims 30 and 31 requiring inclusion of multiple antigens, with Claim 31 requiring a first antigen selected from the group consisting of tetanus toxoid, a nonpathogenic mutant of tetanus toxoid and combinations thereof and a second antigen selected from the group consisting of diphtheria toxoid, a nonpathogenic mutant of diphtheria toxoid and combinations. The only antigen materials identified in *Alonso et al.* that are recited in any of these claims are tetanus toxoid and diphtheria toxoid, which are not the subject of any one of Claims 15 and 17-31. Moreover, Alonso et al. does not disclose any compositions containing multiple antigens, as is the case with both Claims 30 and 31.

Claims 33-35 and 37: Each of dependent Claims 33-35 and 37 requires inclusion of a different particular adjuvant material in the claimed composition. The only material identified in *Alonso et al.* that is recited in any of these claims is chitosan, which is not the subject of any of Claims 33-35 and 37.

Claims 39-43: Each of dependent Claims 39-43 requires the composition to be in a particular form or contained in a particular device. Claims 39 and 40 each requires that the composition be in the form of disperse droplets in a mist, with Claim 40 also requiring that the mist be produced by a nebulizer. Claims 41 and 42 each requires that the composition is contained within a nebulizer actuatable to produce a mist comprising dispersed droplets of the

composition, with Claim 42 also requiring that the nebulizer is a nasal nebulizer. Claim 43 requires that the composition be contained within an injection device actuatable to administer the composition to the host by injection. *Alonso et al.* do mention injection administration, but not in combination with the claimed composition, and *Alonso et al.* do not disclose mist formulations, or containment any composition within a nebulizer.

Claim 44: Claim 44 is directed to a method of packaging and storing the composition of Claim 5, and requires placing the composition in a container when the composition is in the form of a flowable medium and, after the placing, raising the temperature of the composition in the container to convert the composition to the gel form for storage, and wherein the gel form in the container can be converted back to the form of a flowable medium for administration to the host by lowering the temperature of the composition in the container. *Alonso et al.* do not disclose such packaging and storing.

Claims 148 and 149: Claim 148 requires that substantially all of the polyoxyalkylene block copolymer is dissolved in the liquid at some temperature within the temperature range recited in Claim 1, and Claim 149 requires that substantially all of the copolymer and the antigen are dissolved in the liquid at some temperature within the temperature range recited in Claim 1. *Alonso et al.* do not disclose either of these additional features, and *Alonso et al.* teach away from these features through the object of *Alonso et al.* to form drug delivery nanoparticles, which nanoparticles would not be in a dissolved state.

II. Ground 2, 35 U.S.C. §103(a)

The rejection of Claims 1, 4-7, 9-37, 39-44 and 148-149 under 35 U.S.C. § 103(a) as anticipated by *Alonso et al.* in view of *Hale et al.* and *Viegas et al.* is not proper.

As discussed above, *Alonso et al.* disclose two types of compositions, one being drug delivery nanoparticles and the other being an aqueous-based formation medium used during manufacture processing to make the drug delivery nanoparticles. As discussed above, the drug delivery nanoparticles of *Alonso et al.* do not contain 60 to 85 weight percent aqueous liquid and do not exhibit reverse thermal viscosity behavior, as required in appealed Claim 1. Also, the formation medium of *Alonso et al.* does not contain 60 to 85 weight percent aqueous liquid, does not contain from 5 to 33 weight percent polyoxyalkylene block copolymer and does not exhibit reverse thermal viscosity behavior, as required in appealed Claim 1.

The secondary references *Viegas et al.* and *Hale et al.* do not make up for the deficiencies of *Alonso et al.* to render the claimed subject matter obvious for purposes of 35 U.S.C. §103(a).

A. *Viegas et al.*

The following excerpts are quoted from *Viegas et al.* (with the excerpts are numbered for easy reference):

(xv) This invention relates to ophthalmic drug delivery vehicles, drug delivery compositions, and medical devices comprising an aqueous gel. [*Viegas et al.*, at column 1, lines 13-15.]

(xvi) Drug delivery vehicles, drug delivery compositions, and medical devices are

disclosed. The pharmaceutical compositions contain pharmacologically active medicaments useful in providing treatments to ophthalmic areas of the mammalian body requiring pharmacological treatment or requiring the administration of a diagnostic agent. The use of compositions of the invention as medical devices is indicated where an aqueous gel is desirable to cover injured tissue or separate delicate tissues in a manner more gentle than can be accomplished using surgical instruments. [*Viegas et al.*, at column 2, lines 3-13.]

(xvii) In a preferred embodiment, the compositions of the invention can be delivered to the area of the mammalian body requiring treatment as a low viscosity liquid at ambient temperatures which, upon contact with the mammalian body, forms a semisolid gel having a very high viscosity. Because the preferred compositions of the invention are low viscosity liquids at ambient temperatures, they easily pass to various ophthalmic areas insuring maximum contact between exposed tissue and, for instance, the pharmaceutical compositions of the invention . . . A wide variety of polyoxyalkylene copolymers are suitable for the preparation of the compositions of the invention. Generally, it is necessary to adjust the copolymer concentration in aqueous solution so as to obtain the desired sol-gel transition temperature in order that the compositions can be provided as low viscosity liquids at ambient temperature, yet form semisolid gels at mammalian body temperatures . . . The useful copolymers which provide the sol-gel characteristics of the pharmaceutical compositions of the invention are, preferably, polyoxyalkylene block copolymers. [*Viegas et al.*, at column 2, lines 39-68.]

(xviii) It has been found that aqueous pharmaceutical vehicles or medical devices containing a polyoxyalkylene block copolymer, which have the unique feature, in a preferred embodiment, of being liquid at ambient temperatures and transitioning at mammalian body temperatures to a semisolid gel, can be made isotonic or iso-osmotic (or hyperosmotic or hypo-osmotic) and adjusted to the pH of mammalian

body fluids, such as lacrimal tears. [*Viegas et al.*, at column 3, lines 26-35]

(xix) Preferably the drug delivery compositions of the invention will contain from about 0.01% to about 60% by weight of the medicament or pharmaceutical, and generally from about 10 to about 50% of the polymer and from 80% to about 20% water. In special situations, however, the amounts may be varied to increase or decrease the dosage schedule. [*Viegas et al.*, at column 8, lines 20-26.]

1. *Alonso et al.* and *Viegas et al.* are not combinable, because there is no suggestion, teaching or motivation for such combination, and on the contrary such a combination would be contrary to the principles of the teachings of *Alonso et al.*

For references to be combined, there must be some suggestion, teaching or motivation to make the combination, which may be provided from a range of sources, such as flowing from the prior art references themselves, the knowledge of one of ordinary skill in the art, or, in some cases, the nature of the problem to be solved. *In re Dembiczak*, 175 F.3d 994, 999, 50 U.S.P.Q.2d 1614 (Fed. Cir. 1999). As stated in *Dembiczak*:

> [T]he best defense against the subtle but powerful attraction of a hindsight-based obviousness analysis is rigorous application of the requirement for a showing of the teaching or motivation to combine prior art references. [*Dembiczak*, 175 F.3d at 999.]

In the Appealed Office Action, the Examiner made no showing as to any suggestion, teaching or motivation for making the combination of *Alonso et al.* and *Viegas et al.*, but a

review of the two references reveals that the different teachings of the references are actually contrary to making such a combination.

The teachings of *Alonso et al.* and *Viegas et al.* are contrary to each other, and therefore not logically combinable, even though both concern drug delivery compositions. The reason that the teachings are contrary to each other is that the different approaches to drug delivery disclosed by the two references are so fundamentally and structurally different that modifying the drug delivery composition of *Alonso et al.* in the way apparently suggested by the Examiner using the teachings of *Viegas et al.* would be destructive to the fundamental drug delivery concept of *Alonso et al.*

Alonso et al. discloses a drug delivery composition that is in the form of nanoparticles based on chitosan, and optionally containing a polyoxyethylene-polyoxypropylene block copolymer. The nature of the nanoparticles of *Alonso et al.* are in significant contrast to the nature of the drug delivery vehicle of *Viegas et al.*, which concerns an aqueous gel that contains polyoxyalkylene block copolymers, and which in a preferred embodiment disclosed by *Viegas et al.* is provided as a low viscosity liquid at ambient temperatures that upon contact with the mammalian body, forms a semisolid gel having a very high viscosity. (*See*, excerpts (xv)-(xviii) above.) The nanoparticles of *Alonso et al.* and the temperature-sensitive gelling compositions of *Viegas et al.* are structurally very different types of drug delivery approaches, so different that they would not logically be considered as susceptible to combination by those of ordinary skill in the art. This is evident from a recognition that modifying the drug delivery nanoparticles of *Alonso et al.* to contain the temperature-sensitive gelling characteristic of the compositions of *Viegas et al.* would be nonsensical, because the compositions of *Viegas et al.* are in the form of a

low viscosity liquid at ambient temperatures, and if the nanoparticles of *Alonso et al.* were in the form of such a low viscosity liquid at ambient temperatures, then the desired nanoparticles of *Alonso et al.* would cease to exist, which would be inimical to the fundamental nature of the drug delivery composition of *Alonso et al.* as being nanoparticles.

In addition to the drug delivery composition in the form of nanoparticles, *Alonso et al.* also disclose a formation medium that is used as a processing medium in the manufacture process by which the nanoparticles are made. Although not specifically asserted by the Examiner, it might be asserted that one of ordinary skill in the art would consider in combination the formation medium aspect of *Alonso et al.* with the temperature-sensitive gelling drug delivery composition of *Viegas et al.* But considering such a combination would also be nonsensical. The drug delivery compositions of *Viegas et al.* and processing media or *Alonso et al.* for making drug delivery nanoparticles have different purposes, and are simply not analogous. One of ordinary skill in the art considering manufacture processes to make drug delivery nanoparticles, of the type disclosed by *Alonso et al.*, would have no motivation to consider modification of the formation medium of *Alonso et al.* to give it the temperature-sensitive gelling characteristic of the drug delivery composition of the type disclosed by *Viegas et al.* The purpose of the formation medium of *Alonso et al.* is for use as a processing medium to make the drug delivery nanoparticles in a manufacture process, whereas the purpose of the temperature-sensitive gelling aspect of the *Viegas et al.* drug delivery composition is to facilitate drug delivery in the form of a low viscosity liquid that then gels upon contact with the mammalian body after administration. Moreover, there is no indication that the temperature-sensitive gelling characteristic of the drug delivery composition of *Viegas et al.* would directly relate to any perceived problem with or any

potential benefit in relation to use of the formation mediation of *Alonso et al.* for its intended purpose, which is as a process medium for use during a manufacture process to make the drug delivery nanoparticles of *Alonso et al.*

2. Even if *Viegas et al.* were combinable with *Alonso et al.*, the subject matter of Claim 1 would not be obvious from such a combination.

Even if, solely for the sake of argument, *Viegas et al.* and *Alonso et al.*, were assumed to be combinable, such a combination would still not make obvious the subject matter of Claim 1. The discussion above concerning reasons for non-combinability are relevant also with respect to what one of the ordinary skill in the art would make of the combined teachings of *Alonso et al.* and *Viegas et al.*

One of ordinary skill in the art considering *Alonso et al.* and *Viegas et al.* in combination would not find obvious modification of the drug delivery nanoparticles of *Alonso et al.* to include the temperature-sensitive gelling characteristics of the drug delivery composition of *Viegas et al.*, for at least the following reasons:

(1) if the nanoparticles of *Alonso et al.* were modified to have the temperature-sensitive gelling characteristics of the drug delivery composition of *Viegas et al.*, the nanoparticles would be in the form of a low viscosity liquid at ambient temperatures, which would be destructive to the nanoparticulate structure desired by *Alonso et al.* (especially because there is no disclosure in *Alonso et al.* that

would indicate that the nanoparticles of *Alonso et al.* are manufactured at other than ambient temperatures); and

(2) the drug delivery composition of *Viegas et al.* preferably contains 20 to 80% water (and the compositions of Examples 4 and 5 of *Viegas et al.* each contains over 90% buffer solution) and modification of the drug delivery nanoparticles of *Alonso et al.* to contain such large quantities of water would be contrary to the disclosure of *Alonso et al.* that the major constituents of the drug delivery nanoparticles are chitosan and PEO-PPO, and possibly the macromolecular active ingredient.

One of ordinary skill in the art considering a combination of *Alonso et al.* and *Viegas et al.* would not find obvious a modification of the formation medium of *Alonso et al.* to include the temperature-sensitive gelling characteristic of the drug delivery composition of *Viegas et al.* because the purpose of the formation medium of *Alonso et al.* is to serve as a processing medium to manufacture drug delivery nanoparticles, whereas the purpose of the temperature-sensitive gelling characteristic of the drug delivery composition, as disclosed by *Viegas et al.*, is to permit the drug delivery composition to easily pass to various ophthalmic areas. (*See*, excerpt (xvii) above.) There is no reason why one of ordinary skill in the art would find obvious making a modification to a process medium to include a feature (reverse thermal viscosity behavior), the disclosed purpose of which feature is to facilitate drug delivery in a drug delivery composition (liquid at ambient temperature for ophthalmic administration and converting to a gel at body

temperature following such administration) that is fundamentally and structurally different than the drug delivery composition (nanoparticles) made using that process medium.

The Examiner, in paragraph 7, on pages 3-4 of the Appealed Office Action, cites to several cases in support of the following assertion: "Generally, differences in concentration or temperature will not support the patentability of subject matter encompassed by the prior art unless there is evidence indicating such concentration or temperature is critical." Although there may be some dispute about the Examiner's general assertions, in any event the cases cited by the Examiner are not on point with the facts present here.

The cases cited by the Examiner are: *In re Aller*, 220 F.2d 454, 205 U.S.P.Q. 233(CCPA 1955); *In re Peterson*, 315 F.3d 1325, 65 U.S.P.Q.2d 1379 (Fed.Cir. 2003); *In re Hoeschele*, 406 F.2d 1403, 160 U.S.P.Q. 809 (CCPA 1969); and *In re Rose*, 220 F.2d 459, 105 U.S.P.Q. 237 (CCPA 1955). In each of *Aller*, *Peterson* and *Hoeschele*, the claimed subject matter and the prior art subject matter were essentially the same (particular process for making phenol in *Aller*; particular type of nickel-based alloy in *Peterson*; and particular polyurethanes in *Hoeschele*) and the modification to the prior art to find obvious the invention involved mere adjustment of concentrations of reactants and reaction temperature (*Aller*), concentration of composition components (*Peterson*) and proportion of composition components (*Hoeschele*). Unlike the situations in *Aller*, *Peterson*, and *Hoeschele*, in the instant case the subject matter of Claim 1 concerns an antigen delivery composition that exhibits reverse thermal viscosity behavior, whereas the teachings of *Alonso et al.* do not concern essentially the same subject matter, because the delivery composition of *Alonso et al.* is in the quite different form of nanoparticles. The facts of *Aller*, *Peterson* and *Hoeschele* are not analogous to the facts present here. The case

of *Rose* is somewhat different, but is also not analogous to the facts of the present situation. In *Rose*, the invention concerned a particular manner of assembling stacks of lumber composed of lumber of different lengths, and the prior art concerned assembling stacks of a variety of different materials (not all of which were lumber), but there was a commonality in the problem addressed, i.e. how to assemble stacks of things of different size. No such analogous factual situation exists here that is common between the subject matter of the appealed claims and *Alonso et al.* Again, the delivery composition approaches recited in the appealed claims and the drug delivery nanoparticles of *Alonso et al.* are fundamentally and structurally different.

Clearly, *Viegas et al.* do not provide any disclosure that, if combinable with *Alonso et al.*, would cure the deficiency in the teachings of *Alonso et al.*

B. *Hale et al.*

The following excerpts are quoted from *Hale et al.* (with the excerpts are numbered for easy reference):

(xx) Compositions are provided comprising a pharmaceutical agent covalently bonded to one or more charged chemical modifiers through physiologically cleavable bonds to form a pharmaceutical agent-chemical modifier complex, wherein the modifiers enhance the delivery and/or transport of the pharmaceutical agent through membranes, or other biological or physical property of the agent. This invention also provides a pharmaceutical formulation which comprises a pharmaceutically effective amount of a pharmaceutical agent-chemical modifier composition and acceptable physiological carriers or excipients thereof. [*Hale et*

al., at column 3, lines 51-61.]

(xxi) The pharmaceutical agent-modifier complex can be admixed with an acceptable physiological carrier, such as water, aqueous alcohols, propylene glycol, dimethylsulfoxide, to make a composition suitable for contact with the various membranes and transport and delivery through these membranes. [*Hale et al.*, at column 44, lines 26-31.]

(xxii) The pharmaceutical agent-chemical modifier complexes described herein can be administered transdermally. Transdermal administration typically involves the delivery of a pharmaceutical agent for percutaneous passage of the drug into the systemic circulation of the patient . . . A variety of types of transdermal patches will find use in the methods described herein . . . For passive delivery systems, the rate of release is typically controlled by a membrane placed between the reservoir and the skin, by diffusion from a monolithic device, or by the skin itself serving as a rate-controlling barrier in the delivery system . . . Suitable permeable membrane materials may be selected based on the desired degree of permeability, the nature of the complex, and the mechanical considerations related to constructing the device. Exemplary permeable membrane materials include a wide variety of natural and synthetic polymers, such as polydimethylsiloxanes (silicone rubbers), ethylenevinylacetate copolymer (EVA), polyurethanes, polyurethane-polyether copolymers, polyethylenes, polyamides, polyvinylchlorides (PVC), polypropylenes, polycarbonates, polytetrafluoroethylenes (PTFE), cellulosic materials, e.g., cellulose triacetate and cellulose nitrate/acetate, and hydrogels, e.g., 2-hydroxyethylmethacrylate (HEMA). [*Hale et al.*, at column 46, line 27 through column 47, line 57, under the heading "Transdermal Delivery"]

(xxiii) [T]he methods of the present invention are also applicable to the enhanced transport and delivery of pharmaceutical agents through mucosal membranes,

> such as gastrointestinal, sublingual, buccal, nasal, pulmonary, vaginal, corneal, and ocular membranes . . . For delivery to the nasal and/or pulmonary membranes, typically an aerosol formulation will be employed. The term "aerosol" includes any gas-borne suspended phase of the pharmaceutical agent-chemical modifier complex which is capable of being inhaled into the bronchioles or nasal passages. Specifically, aerosol includes a gas-borne suspension of droplets of the compounds of the instant invention, as may be produced in a metered dose inhaler or nebulizer, or in a mist sprayer. Aerosol also includes a dry powder composition of the pharmaceutical agent-chemical modifier complex suspended in air or other carrier gas, which may be delivered by insufflation from an inhaler device, for example. [*Hale et al.*, at column 52, line 39 through column 53, line 25, under the heading "Transmucosal Delivery".]

1. *Alonso et al.* and *Hale et al.* are not combinable, because there is no suggestion, teaching or motivation for such combination.

In the Appealed Office Action, the Examiner has not made a showing as to any suggestion, teaching or motivation that would lead one of ordinary skill in the art to combine the teachings of *Alonso et al.* and *Hale et al.*, and from a review of the references, no such suggestion, teaching or motivation is apparent. As noted above, *Alonso et al.* disclose a drug delivery composition that is in the form of nanoparticles based on chitosan, and optionally containing a polyoxyethylene-polyoxypropylene block copolymer. In contrast, the drug delivery compositions of *Hale et al.* are based on covalently bonding a modifier to a pharmaceutical agent to form a pharmaceutical agent-chemical modifier complex to facilitate drug delivery. The two references concern different approaches to formulation of drug delivery compositions, and there is no teaching, suggestion or motivation for one of ordinary skill in the art to combine the

teachings concerning these quite different approaches to formulation of drug delivery compositions.

2. Even if *Hale et al.* were combinable with *Alonso et al.*, the subject matter of Claim 1 would not be obvious from such a combination.

The disclosure of *Hale et al.* concerns modifying pharmaceutical agents with certain chemical modifiers that are covalently bonded to the pharmaceutical agent to form a pharmaceutical agent-chemical modifier complex to provide enhanced delivery or transport of the pharmaceutical agent, such as through membranes. (*See*, excerpt (xx) above.) *Hale et al.* further disclose, in a general fashion, that the pharmaceutical agent-modifier complex may be admixed with an acceptable physiological carrier, and provides some examples. (*See*, excerpt (xxi) above.) Also, in Table 2 presented in columns 15 and 16 of *Hale et al.*, "vaccines" is listed as an example of a peptide or protein drug that could be used as the pharmaceutical agent.

Nowhere, however, do *Hale et al.* disclose any particulars in relation to formulation or delivery of compositions including vaccines. Also, *Hale et al.* do not disclose compositions including non-alum adjuvants for antigens, or polyoxyalkylene block copolymers, or exhibiting reverse thermal viscosity behavior.

In the Appealed Office Action, the Examiner asserted that *Hale et al.* teach a composition comprising a therapeutic composition "comprising a therapeutic agent imbedded with copolymers of polyethylenes that is produced as an inhaler or nebulizer or in a mist of spryer [sic] suitable for the transmucosal delivery, wherein the composition may be produced as a dry

powder or an inhaler or a nebulizer or a mist sprayer." (Appealed Office Action, paragraph 6, page 3, citations omitted.) The portion of *Hale et al.* cited to by the Examiner in relation to "therapeutic agent imbedded with copolymers of polyethylenes" is included in excerpt (xxiii) above, and the portion of *Hale et al.* cited by the Examiner in relation to "inhaler or nebulizer . . . mist . . . dry powder", etc. is included in excerpt (xxiii) above. These different portions of *Hale et al.* cited by the Examiner are not combinable as indicated by the Examiner, because they concern very different routes of drug administration, but in any event these teachings of *Hale et al.* do not appear to be particularly relevant to the subject matter of the appealed Claims.

The portion of *Hale et al.* cited by the Examiner concerning a "therapeutic agent imbedded with copolymers of polyethylenes" is part of a discussion by *Hale et al.* concerning possible membrane materials for use in a passive delivery transdermal patch across which the pharmaceutical agent-modifier complex of *Hale et al.* is transported during passive transdermal administration. (*See*, excerpt (xxii) above.) Moreover, the reference made by *Hale et al.* to "polyethylenes" is not to copolymers as asserted by the Examiner, and polyethylenes are simple hydrocarbon chain polymers whereas the polyoxyalkylene blocks of polyoxyalkylene block copolymers, which are recited in the appealed Claims, are polyether segments. (*See*, the Application description, at page 17, line 6 through page 18, line 33.) It is not apparent why disclosure by *Hale et al.* concerning transdermal delivery of the pharmaceutical agent-modifier complex across a polyethylene membrane would even be relevant to the subject matter of the appealed Claims.

The portion of *Hale et al.* cited by the Examiner concerning "inhaler or nebulizer . . . mist . . . dry powder", etc. is part of a discussion by *Hale et al.* concerning possible aerosolization

of the pharmaceutical agent-modifier complex for delivery to nasal and/or pulmonary membranes in an aerosol formulation. (*See*, excerpt (xxiii) above.) This portion of *Hale et al.* is completely separate from and is incompatible with the other portion of *Hale et al.* cited by the Examiner concerning transdermal delivery from a transdermal patch. Moreover, to the extent that the disclosure in *Hale et al.* concerning delivery of the pharmaceutical agent-chemical modifier complex of *Hale et al.* is of any relevance to the Appealed claims, it would only be with respect to the particular features recited in dependent Claims 39-42, which are discussed elsewhere.

Clearly, *Hale et al.* do not provide any disclosure that, if combinable with *Alonso et al.*, would cure the deficiency in the teachings of *Alonso et al.* and *Viegas et al.*

C. Dependent claims recite one or more additional feature that, in combination with the limitations of Claim 1, further distinguish over *Alonso et al.* in view of *Viegas et al.* and *Hale et al.*

With respect to appealed dependent Claims 4-7, 9-31, 33-37, 39-44, 148 and 149, these claims are also not obvious over *Alonso et al.* in view of *Viegas et al.* and *Hale et al.*, for the same reasons as stated above with respect to independent Claim 1. Moreover, each of these dependent Claims contains one or more limitations in addition to the limitations recited in appealed independent Claim 1, which additional limitation or limitations, in combination with the limitations of Claim 1, further distinguish over *Alonso et al.*, *Viegas et al.* and *Hale et al.*, including any purported combinations of those references.

Claims 4-7: As discussed above, each of dependent Claims 4-7 requires that the

composition exhibit particular viscosity behavior in that the composition is in the form of a flowable medium at a first, lower temperature, and in the form of a gel at a second, higher temperature. The teachings of *Viegas et al.* do concern making reverse thermal gelation compositions, but not in the combination of the antigen delivery composition with specific gelation characteristics as required in Claims 4-7, and *Hale et al.* do not even disclose reverse thermal viscosity behavior. Furthermore, the processing disclosed in *Alonso et al.* to make the drug delivery nanoparticles of *Alonso et al.* is based on a crosslinking reaction to spontaneously precipitate and form the nanoparticles, which crosslinking reaction would be expected to interfere with the viscosity behavior disclosed by *Viegas et al.*, so that one of ordinary skill in the art would not consider making the modification to compositions of *Alonso et al.*, and even if one of ordinary skill in the art did consider the modification, there would be no reasonable expectation of success.

Claims 9-14: Each of dependent Claims 9-14 requires a feature recited in Claim 9 that the polyoxyalkylene block copolymer comprises at least one block each of a first polyoxyalkylene and a second polyoxyalkylene, with claims 11-14 requiring particular formulae for the polyoxyalkylene block copolymer. *Hale et al.* do not disclose polyoxyalkylene block copolymers, and Viegas et al, while disclosing a variety of polyoxyalkylene block copolymers, do not alone or in combination with *Alonso et al.* teach, suggest or motivate a modification to the teaching of *Alonso et al.* for use of such polyoxyalkylene block copolymers in the particular combinations recited in any of Claims 9-14.

Claims 16 and 17-31: Each of dependent Claims 15-31 requires inclusion of particular antigen material in the claimed composition, with Claims 30 and 31 requiring inclusion of

multiple antigens, as noted above. The only antigen materials identified in *Alonso et al.* that are recited in any of these Claims are tetanus toxoid and diphtheria toxoid, which are not the subject of any of Claims 15 or 17-29, and *Alonso et al.* do not disclose compositions including multiple different antigens, as required of Claims 29 and 30. *Viegas et al.* do not mention antigens at all, being focused on particularities associated with ophthalmic delivery. *Hale et al.* lists "vaccines" generally in an extensive listing extending from column 11 to column 23 of *Hale et al.* of potential pharmaceutical agents for use in the pharmaceutical agent-chemical modifier complex disclosed by *Hale et al.* for drug delivery across membranes. Such a general listing of "vaccines," even if *Hale et al.* were combinable with *Alonso et al.*, would not render obvious the particular antigen materials listed in any of Claims 5 or 17-29 or the use of multiple antigens as required in Claims 30 and 31.

Claims 33-35 and 37: Each of dependent Claims 33-35 and 37 requires inclusion of a different particular adjuvant material in the claimed composition. The only material identified in *Alonso et al.* that is recited in any of these claims is chitosan, and the inclusion of chitosan was not for the purpose of acting as an adjuvant for antigen delivery, even if the material might serve that purpose when the composition of *Alonso et al.* includes the disclosed tetanus toxoid or diphtheria toxoid. *Viegas et al.* do not mention antigens, since *Viegas et al.* are focused on particularities associated with ophthalmic delivery, and understandably, *Viegas et al.* also provide no disclosure concerning adjuvants for antigen delivery. As noted above, *Hale et al.* only generally mentions "vaccines" in an extensive listing of potential pharmaceutical agents for use in the pharmaceutical agent-chemical modifier complex of *Hale et al.* *Hale et al.* do not discuss adjuvants for antigen delivery, and even if *Hale et al.* were combinable with *Alonso et al.*, such a

combination would not render obvious the particular non-alum adjuvants recited in any of Claims 33-35 and 36.

Claims 39-43: Each of dependent Claims 39-43 requires the composition to be in a particular form or contained in a particular device. Claims 39 and 40 each requires that the composition be in the form of disperse droplets in a mist, with Claim 40 also requiring that the mist be produced by a nebulizer. Claims 41 and 42 each requires that the composition is contained within a nebulizer actuatable to produce a mist comprising dispersed droplets of the composition, with Claim 42 also requiring that the nebulizer is a nasal nebulizer. Claim 43 requires that the composition be contained within an injection device actuatable to administer the composition to the host by injection. *Alonso et al.* do mention injection administration, but not in combination with the claimed composition, and *Alonso et al.* do not disclose mist formulations, or containment of compositions in a nebulizer. The Examiner cited to *Hale et al.* in relation to its teachings concerning nasal/pulmonary delivery of the pharmaceutical agent-chemical modifier complex of *Hale et al.* in an aerosol formulation. The drug delivery composition of *Hale et al.*, which is in the form of the pharmaceutical agent-chemical modifier complex is significantly different than the drug delivery nanoparticles of *Alonso et al.*, and there is no showing of a teaching, suggestion or motivation that would lead one of ordinary skill in the art to attempt to modify the drug delivery nanoparticles of *Alonso et al.* to have them administered in such an aerosol formulation. *Viegas et al.* do not disclose formulation for administration via injection or in mist form the disclosed drug delivery composition of *Viegas et al.* that is designed for ophthalmic delivery.

Claim 44: Claim 44 is directed to a method of packaging and storing the composition of

Claim 5, and requires placing the composition in a container when the composition is in the form of a flowable medium and, after the placing, raising the temperature of the composition in the container to convert the composition to the gel form for storage, and wherein the gel form in the container can be converted back to the form of a flowable medium for administration to the host by lowering the temperature of the composition in the container. None of *Alonso et al.*, *Viegas et al.* or *Hale et al.* mention packaging or storing any product, let alone the composition recited in appealed Claim 5 or the specifics recited in appealed Claim 44.

Claims 148 and 149: Claim 148 requires that substantially all of the copolymer is dissolved in the liquid at some temperature within the temperature range recited in Claim 1, and Claim 149 requires that substantially all of the copolymer and the antigen are dissolved in the liquid at some temperature within the temperature range recited in Claim 1. As noted above, *Alonso et al.* do not disclose these additional features, and teaches away from these features through the object of *Alonso et al.* to form drug delivery nanoparticles, which nanoparticles would not be in a dissolved state. *Viegas et al.* do not disclose the use of antigens, and accordingly do not disclose compositions with dissolved antigens. *Hale et al.* do not disclose polyoxyalkylene block copolymers at all and, although *Hale et al.* do generally disclose "vaccines" as a potential for the pharmaceutical agent in an extensive listing, *Hale et al.* do not disclose compositions including dissolved antigens.

III. Ground 3, 35 U.S.C. § 112, First Paragraph

The rejection of Claims 1, 31, 33-44 and 148-149 under 35 U.S.C. § 112, first paragraph, based on an assertion of a lack of an enabling disclosure is not proper.

To be enabling under 35 U.S.C. § 112, first paragraph, a description of the invention must be sufficient to "enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same . . ." A description may be enabling even though one of ordinary skill in the art would have to resort to experimentation to make or use the invention, provided that the extent of the experimentation required is not undue. *United States v. Telectronics*, 857 F.2d 778, 785, 8 U.S.P.Q.2d 1217 (Fed. Cir. 1988). Moreover, an applicant is not required to provide a description of every conceivable embodiment of an invention. *Telectronics*, 857 F.2d at 786. As stated in *Ex parte Forman*, 230 U.S.P.Q. 546 (Bd. Pat. App. & Int. 1986):

> The determination of what constitutes undue experimentation in a given case requires the application of a standard of reasonableness, having due regard for the nature of the invention and the state of the art. The test is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed to enable the determination of how to practice a desired embodiment of the invention claimed. [Citations omitted, emphasis added.]

The Examiner cited to *In re Wands*, 858 F.2d 731, 8 U.S.P.Q.2d 1400 (Fed. Cir. 1988) concerning factors for consideration in determining whether undue experimentation would be required. Concerning these factors, *Wands* specifically states:

> They include (1) the quantity of experimentation necessary, (2) the amount of direction or guidance presented, (3) the presence or absence of working examples, (4) the nature of

> the invention, (5) the state of the prior art, (6) the relative skill of those in the art, (7) the predictability or unpredictability of the art, and (8) the breadth of the claims. [*Wands*, 858 F.2d at 737.]

Each of the factors identified by *Wands* is discussed below in relation to the appealed independent Claim 1 rejected under 35 U.S.C. § 112, first paragraph.

Nature Of The Invention; Breadth Of Claims: The nature of the invention of Claim 1 may be characterized as concerning a manner of formulating antigens for delivery, represented as a delivery composition that includes a combination of antigen, non-alum adjuvant, polyoxyalkylene block copolymer and aqueous liquid, with the aqueous liquid and the polyoxyalkylene block copolymer proportioned to impart reverse-thermal viscosity behavior to the composition. Importantly, the invention of the appealed Claims does not claim any antigens, adjuvants or copolymers, *per se*, and is not premised on the development of any new antigens, adjuvants or copolymers. Because of the nature of the claimed invention involves formulation of materials, rather than development of new chemical compounds, many of the issues involved with generically extending principles associated with synthesis of a new chemical compound simply do not apply to the claimed invention. The breadth of the claims is not limited to only those particular antigens, polyoxyalkylene block copolymers or non-alum adjuvants specifically disclosed in the Application or included in a working example, but the claims are nevertheless, narrowly drawn to only a particular type of antigen delivery composition comprising those components within particular compositional ranges and with the composition exhibiting reverse thermal viscosity behavior. The scope of the claims is readily comprehensible to those skilled in the art and the claims do not foreclose research into and development of new antigens or

adjuvants, but rather impacts only a narrow technical scope related to a particular formulation approach for preparing compositions for delivery of antigens.

Amount Of Direction And Guidance Presented; Presence Or Absence Of Working Examples: The description portion of the specification of the Application provides significant direction and guidance. In the application, discussion concerning exemplary possible antigens for potential inclusion in the claimed composition is presented at page 14, line 14 through page 15, line 22; discussion concerning exemplary possible polyoxyalkylene block copolymers for potential inclusion in the claimed composition is presented at page 17, line 8 through page 18, line 33; discussion concerning exemplary possible non-alum adjuvants for potential inclusion in the claimed composition is presented at page 20, lines 7-25; discussion concerning use of an aqueous liquid as a liquid vehicle for the claimed composition is presented at page 7, lines 24-26, page 9, lines 2-4, and page 21, lines 7-10; discussion concerning the reverse thermal viscosity behavior of the composition, including that involving reverse thermal gelation, is presented at page 7, line 27 through page 8, line 23, page 10, lines 6-11, page 16, line 12 through page 17, line 5; discussion concerning component concentrations, other than included in the discussions noted above, is presented at page 20, lines 25-29; and discussion concerning use of the composition, including administration of the composition to patients, is presented at page 8, line 17 through page 9, line 4, page 9, line 32 through page 10, line 23, page 14, lines 14-21, and page 22 line 24 through page 24, line 7. This significant direction and guidance on how to make and use the claimed composition is supplemented by working examples presented in the Application. A total of 10 working examples are presented on pages 25-33 of the Application specification, showing preparation of examples of the claimed composition using Pluronic® F127 polymer as

the polyoxyalkylene block copolymer, chitosan as the non-alum adjuvant and tetanus toxoid, chicken ovalbumin or diphtheria toxoid as the antigen. These working examples also include demonstration of the use of the example compositions in mice studies with administration being made intranasally, subcutaneously or intraperitoneally, with the example compositions administered as a prime administration or a boost administration, and with immune response data provided relative to various comparison compositions. Reference is also made to the data presented in the Coeshott Declaration, including the exhibits thereto, included in Appendix B of this Appeal Brief, showing additional example compositions formulated and tested in mice studies, in similar manner to the working examples provided in the Application, with the same antigens as the working examples (tetanus toxoid and diphtheria toxoid) and an additional antigen (recombinant anthrax protective antigen) and with the same non-alum adjuvant (chitosan) and an additional non-alum adjuvant (CpG dinucleotide motifs). The additional examples presented in the Coeshott Declaration are referenced here not as being a part of the enabling disclosure of the Application, but as evidence concerning the validity of the direction and guidance provided by the Application description, including the direction and guidance provided by the 10 working examples presented in the Application description.

Quantity Of Experimentation: The quantity of experimentation required to make the claimed composition is only of a type that would be routine in the formulation of antigen delivery compositions. All that is required to make the composition is a simple mixing of the four ingredients listed in the claims: antigen, aqueous liquid, polyoxyalkylene block copolymer and non-alum adjuvant. For any such mixture, the mixture can be easily tested for the presence of reverse thermal viscosity behavior over some temperature range within 1 °C to 37 °C by simply

testing the viscosity at various temperatures while the temperature of the mixture is raised from 1 °C to 37 °C. Use of any particular composition for administration to patients would require only normal testing, such as for safety and dosing, as would be performed for any pharmaceutical product.

Predictability Or Unpredictability Of The Art: Making the claimed antigen delivery composition involves only simple mixing of components, and is hardly in an unpredictable art in that respect. The claimed antigen delivery composition does not involve synthesis of antigens, adjuvants or polyoxyalkylene block copolymers, and does not involve the unpredictability associated with extending chemical synthesis techniques to undisclosed species of a generic claim to newly created chemical compounds. There is always some unpredictability as to how any particular delivery composition will perform upon administration during use, but such unpredictability is routinely handled in the art of drug delivery through routine testing, such as for safety and dosing, as discussed above.

State Of The Prior Art: The state of the prior art in relation to the nature of polyoxyalkylene block copolymers and using the same to prepare reverse thermal viscosity formulations is well developed. For example, reference is again made to *Newman et al.* and *Cha et al.* as an indication of the level of understanding in the prior are concerning preparing formulations with polyoxyalkylene block copolymers. Additionally, reference is also made to U.S. Patent No. 5,861,174 by Stratton et al. as further evidence of the state of the art with respect to preparing formulations with polyoxyalkylene block copoloymers. These are only some examples of the many references of record that discuss polyoxyalkylene block copolymers.

Relative Skill Of Those In The Art: The relative skill of those involved with formulating

of antigen delivery compositions is high, as it is generally in pharmaceutical arts. In that regard, the technical qualifications set forth for in Exhibit A of the Coeshott Declaration, included in Appendix B of this Appeal Brief, are representative of this high level of skill in the art.

On balance, these factors weigh strongly in favor of a finding that one of skill in the art would not have to undertake undue experimentation to make and use the full breadth of the subject matter set forth in appealed Claim 1. The nature of the claimed invention is directed to a combination of components in a particular type of antigen delivery composition, and is not directed to development of new antigens, new polyoxyalkylene block copolymers or new adjuvants; and the breadth of the claims is narrowly tailored to the nature of the invention and does not preempt development of new antigens, adjuvants, or polyoxyalkylene block copolymers. A substantial amount of direction and guidance is presented on how to make and use the claimed subject matter, including 10 working examples. The art does not involve a high level of unpredictability, there is a high level of skill in the art, the state of the prior art concerning the components to be combined in the claimed composition is well developed, and the quantity of experimentation is not large and is of a routine type.

The Examiner's position in relation to the "thousands" of polyoxyalkylene copolymer molecules that would have to be tested in asserting lack of enablement is misplaced. The Examiner's position seems to be that enablement would require that the Application must definitively identify each and every polyoxyalkylene block copolymer that would and would not be formulatable with the other components recited in the claims to make the claimed composition that exhibits reverse thermal viscosity behavior. Such a requirement would place an unreasonable and unwarranted burden on an applicant, and is not consistent with the case law

considering the nature of the claimed invention as being directed only to a combination of antigen, non-alum adjuvants and polyoxyalkylene block copolymers in a specific formulation for delivery of the antigen, and not as being directed to a development of any new antigens, adjuvants or polyoxyalkylene block copolymers. The present invention should not be unduly restricted relative to the nature of the invention as a combination of components that are not themselves the subject of development.

In that regard, the present situation is not unlike that presented in the case of *In Re Fuetterer*, 319 F.2d 259, 138 U.S.P.Q. 217 (CCPA 1963). In *Fuetterer*, the claims at issue recited a rubber stock for producing tire treads including "a mixture of a non-adhesive protein and a carbohydrate which mixture is substantially insoluble in cold water" and including an "inorganic salt that is capable of holding a mixture of said carbohydrate and protein in colloidal suspension in water." *Fuetterrer*, 319 F.2d at 260. The Patent Office rejected the claims, and the Patent Office Board of Appeals affirmed the rejection on grounds that the claims were too broadly drawn because only four of the large number of possible inorganic salts were disclosed in the application. The CCPA overturned the rejection stating that the specification need not set forth all salts which could operate in the claimed combination. The court in *Fuetterrer* rejected the Patent Office's assertion that identifying usable salts would involve undue experimentation and specifically stated:

> Applicant's invention is the *combination* claimed and not the discovery that certain inorganic salts have colloidal suspending properties. We see nothing in the patent law which requires applicant to discover which of all those salts have such properties and which will function properly in his combination. The invention description clearly

> indicates that any organic salt which has such properties is usable in his combination. If others in the future discover that inorganic salts additional to those enumerated do have such properties, it is clear applicant will have no control over them per se, and equally clear his claims should not be so restricted that they can be avoided merely by using some inorganic salt not named by applicant in his disclosure. The only "undue burden" which is apparent to us in the instant case is that which the patent office has attempted to place on the applicant. The patent office would require him to do research on "literally thousands" of inorganic salts and determine which of these are suitable for incorporation into his claimed combination, apparently forgetting that he has not invented, and is not claiming colloidal suspending agents but tire tread stock composed of a combination of rubber and other ingredients. [*Fuetterer*, at 319 F.2d 265, emphasis as in original.]

Likewise, the instant invention set forth in the appealed claims is not premised on the discovery of new antigens, polyoxyalkylene block copolymers or adjuvants. Rather, the claimed composition involves a specific recited combination of antigen, adjuvant, polymer and liquid components in an antigen delivery composition formulated to exhibit reverse thermal viscosity behavior. Consistent with the holding of the CCPA in Fuetterer, claims to the present invention should not be unduly restricted in a manner to permit others to easily circumvent the claims simply because the applicant has not identified in the Application description every specific polyoxyalkylene block copolymer that might or might not be formulatable to impart the required reverse thermal viscosity behavior. Again, the claims are not directed to new antigens, adjuvants or polyoxyalkylene block copolymers, *per se*, but rather only to a specific combination of those materials for delivery of antigens.

The disclosure is enabling for the composition recited in the appealed Claims.

CONCLUSION

The rejections stated in the Final Office Action are unsupportable, and it is respectfully requested that the rejections be reversed, and the application proceed to issuance.

Respectfully submitted,

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Date: May 1, 2006.

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APPENDIX A
CLAIMS

1. (Appealed) A composition for delivery of an antigen for stimulation of an immune response when administered to a host, the composition comprising:

an antigen, a polyoxyalkylene block copolymer and an aqueous liquid;

the polyoxyalkylene block copolymer being biocompatible, not having toxic or injurious effects on biological function in the host when the composition is administered;

wherein, the composition is formulated with relative proportions of the liquid and the copolymer so that the copolymer interacts with the liquid to impart reverse thermal viscosity behavior to the composition, so that the viscosity of the composition increases when the temperature of the composition increases over some temperature range within 1 °C to 37 °C; and

wherein, the composition further comprises an additive enhancing the immune response when the composition is administered to the host, the additive being an adjuvant other than alum; and;

wherein, the liquid comprises from 60 weight percent to 85 weight percent of the composition, the antigen comprises from 0.0001 weight percent to 5 weight percent of the composition, the copolymer comprises from 5 weight percent to 33 weight percent of the composition and the additive comprises from 0.01 weight percent to 10.0 weight percent of the composition.

2. (Cancelled)

3. (Cancelled)

4. (Appealed) The composition of Claim 1, wherein the composition is in the form of a flowable medium when the composition is at a first temperature in the temperature range and

the composition is in a gel form when the composition is at a second temperature in the temperature range, the second temperature being higher than the first temperature.

5. (Appealed) The composition of Claim 4, wherein the first temperature is in a range of from 1 °C to 20 °C.

6. (Appealed) The composition of Claim 4, wherein the first temperature is in a range of from 1 °C to 20 °C and the second temperature is in a range of from 25° C to 37 °C.

7. (Appealed) The composition of Claim 4, wherein the copolymer is substantially all dissolved in the liquid when the composition is at the first temperature, and at least a portion of the copolymer comes out of solution in the liquid when the temperature of the composition is raised from the first temperature to the second temperature.

8. (Cancelled)

9. (Appealed) The composition of Claim 1, wherein the polyoxyalkylene block copolymer comprises at least one block of a first polyoxyalkylene and at least one block of a second polyoxyalkylene.

10. (Appealed) The composition of Claim 9 wherein the first polyoxyalkylene is polyoxyethylene and the second polyoxyalkylene is polyoxypropylene.

11. (Appealed) The composition of Claim 10, wherein the polyoxyalkylene block copolymer has the formula:

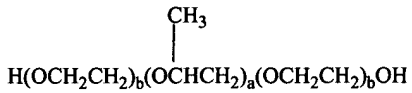
$HO(C_2H_4O)_b(C_3H_6O)_a(C_2H_4O)_bH$

wherein a and each b are independently selected integers.

12. (Appealed) The composition of Claim 11, wherein the $(C_2H_4O)_b$ blocks together comprise at least 70 weight percent of the polyoxyalkylene block copolymer.

13. (Appealed) The composition of Claim 11 wherein a is between 15 and 80 and each b is independently between 50 and 150.

14. (Appealed) The composition of claim 10, wherein the polyoxyalkylene block copolymer has the formula:



wherein a is 20 to 80 and each b is independently 15 to 60.

15. (Appealed) The composition of Claim 1, wherein the antigen is selected from the group consisting of bacteria, protozoa, fungus, hookworm, virus and combinations thereof.

16. (Appealed) The composition of Claim 1, wherein the antigen is selected from the group consisting of tetanus toxoid, diphtheria toxoid, a non-pathogenic mutant of tetanus toxoid, a non-pathogenic mutant of diphtheria toxoid and combinations thereof.

17. (Appealed) The composition of Claim 1, wherein the antigen is from Bordatella pertussis.

18. (Appealed) The composition of Claim 1, wherein the antigen is from influenza virus.

19. (Appealed) The composition of Claim 1, wherein the antigen is from M. tuberculosis.

20. (Appealed) The composition of Claim 1, wherein the antigen immunizes against a childhood illness.

21. (Appealed) The composition of Claim 1, wherein the antigen is from rotavirus.

22. (Appealed) The composition of Claim 1, wherein the antigen is selected from the group consisting of a polysaccharide, a peptide mimetic of a polysaccharide, an antigen from Neisseria meningitiditis, an antigen from Streptococcus pneumoniae and combinations thereof.

23. (Appealed) The composition of Claim 1, wherein the antigen is from Epstein-Barr virus.

24. (Appealed) The composition of Claim 1, wherein the antigen is from Hepatitis C virus.

25. (Appealed) The composition of Claim 1, wherein the antigen is from HIV.

26. (Appealed) The composition of Claim 1, wherein the antigen comprises a molecule involved in a mammalian reproductive cycle.

27. (Appealed) The composition of Claim 1, wherein the antigen is HCG.

28. (Appealed) The composition of Claim 1, wherein the antigen is a tumor-specific antigen.

29. (Appealed) The composition of Claim 1, wherein the antigen is from a blood-borne pathogen.

30. (Appealed) The composition of Claim 1, wherein the antigen is a first antigen and the composition comprises a second antigen.

31. (Appealed) The composition of Claim 30, wherein the first antigen is selected from the group consisting of tetanus toxoid, a nonpathogenic mutant of tetanus toxoid and combinations thereof; and

the second antigen is selected from the group consisting of diphtheria toxoid, a nonpathogenic mutant of diphtheria toxoid and combinations thereof.

32. (Cancelled)

33. (Appealed) The composition of claim 1, wherein the adjuvant comprises dimethyl dioctadecyl ammonium bromide (DDA).

34. (Appealed) The composition of Claim 1, wherein the adjuvant comprises a CpG motif.

35. (Appealed) The composition of Claim 1, wherein the adjuvant comprises a cytokine.

36. (Appealed) The composition of claim 1, wherein the adjuvant comprises chitosan material.

37. (Appealed) The composition of claim 36, wherein the adjuvant comprises N,O-carboxymethyl chitosan.

38. (Cancelled)

39. (Appealed) The composition of Claim 1, wherein the composition is in the form of disperse droplets in a mist.

40. (Appealed) The composition of Claim 39, wherein the mist is produced by a nebulizer.

41. (Appealed) The composition of Claim 1, wherein the composition is contained within a nebulizer actuatable to produce a mist comprising dispersed droplets of the composition.

42. (Appealed) The composition of Claim 40, wherein the nebulizer is a nasal nebulizer.

43. (Appealed) The composition of claim 1, wherein the composition is contained within an injection device that is actuatable to administer the composition to the host by injection.

44. (Appealed) A method of packaging and storing the composition of claim 5, comprising placing the composition in a container when the composition is in the form of a flowable medium and, after the placing, raising the temperature of the composition in the container to convert the composition to the gel form for storage, wherein the gel form in the container can be converted back to the form of a flowable medium for administration to the host by lowering the temperature of the composition in the container.

45-147 (Cancelled)

148. (Appealed) The method of Claim 1, wherein substantially all of the copolymer is dissolved in the liquid at some temperature within the temperature range.

149. (Appealed) The method of Claim 1, wherein substantially all of the copolymer and the antigen are dissolved in the liquid at some temperature within the temperature range.

150-197. (Withdrawn)

APPENDIX B
EVIDENCE

U.S. Patent Documents:

1. US 5,300,295 by Viegas et al. Entered by the Examiner on a Notice of References Cited (PTO-892) accompanying an Office Action dated April 5, 2004. (Reference referred to in this Appeal Brief as "*Viegas et al.*").
2. US 5,607,691 by Hale et al. Entered by the Examiner on a Notice of References Cited (PTO-892) accompanying the Appealed Office Action dated April June 27, 2005. (Reference referred to in this Appeal Brief as "*Hale et al.*").
3. US 5,702,717 by Cha et al. Listed on Form PTO-1449 as part of Information Disclosure Statement filed August 27, 2001. A signed copy of the Form PTO-1449 signed by the Examiner was included with an Office Action dated October 22, 2002. (Reference referred to in this Appeal Brief as "*Cha et al.*").
4. US 5,861,174 by Stratton et al. Listed on Form PTO-1449 as part of Information Disclosure Statement filed August 27, 2001. A signed copy of the Form PTO-1449 signed by the Examiner was included with an Office Action dated October 22, 2002.

Foreign Patent Documents:

5. EP 0 860 166 A1 by Alonso et al. Entered by the Examiner on a Notice of References Cited (PTO-892) accompanying the Appealed Office Action dated April June 27, 2005. (Reference referred to in this Appeal Brief as "*Alonso et al.*").

Non-Patent Technical Papers:

6. Newman, M. J. Balasubramanian M., and Todd C.W, "Development of adjuvant-active nonionic block copolymers," Advanced Drug Delivery Reviews 32 (1998), pp. 199-223. Listed on Form PTO-1449 as part of Information Disclosure Statement filed October 28, 2003. A copy of the Form PTO-1449 signed by the Examiner was included with an Office Action dated January 28, 2004. (Reference referred to in this Appeal Brief as "*Newman et al.*").

Other Evidence:

7. Rule 132 Declaration of Claire M. Coeshott, dated April 22, 2003, including Appendices; acknowledged by the Examiner in an Office Action dated July 15, 2003, paragraph 1, and in an Office Action dated April 5, 2004, paragraph 1, (referred to as "Coeshott Declaration").

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(54) APPLICATION OF NANOPARTICLES BASED ON HYDROPHILIC POLYMERS AS PHARMACEUTICAL FORMS

(57) Application of nanoparticles based on hydrophilic polymers as, pharmaceutical forms for the administration of active macromolecules. The nanoparticles (having a nanometric size and a hydrophilic character), also called nanospheres or latex, are colloidal systems comprised of the combination of hydrophilic polymers and an active ingredient having a high molecular weight (active macromolecule, molecular weight higher than 1000 daltons). The hydrophilic polymers are the chitosane (an aminopolysaccharide) or its derivatives and the polyoxyethylene or its derivatives. The association of the active macromolecule to said nanoparticles takes place in an aqueous phase without having to use organic solvents or auxiliary toxic substances. The active ingredient charge capacity of the nanoparticles is extremely high and additionally said charge is released in a controlled and time extended way. Additionally, said nanoparticles have a positive surface electric charge which the intensity may vary in relation to its composition.

EP 0 860 166 A1

Description

Application of nanoparticles based on hydrophilic polymers as pharmaceutical forms for the administration of bioactive molecules.

The major constituents of these nanoparticles are two hydrophilic polymers: chitosan, which has a positive charge, and poly(oxyethylene), which has a non-ionic character. The active ingredient, which may be also a major constituent of these nanoparticles, is an antigenic or therapeutic macromolecule (peptide, protein, oligonucleotide, RNA, DNA...). The electrical charge of these colloidal particles can vary, depending on the ratio of the two hydrophilic polymers, from a highly positive value to a near zero value. The size of the nanoparticles can be modulated as well, from few nanometers to a few microns, by adequately selecting the preparation conditions.

Chitosan is a natural cationic polymer produced by deacetilation of the polysaccharide chitin which is obtained from crustacean shells. Chitosan is available in the market in a variety of forms (with different molecular weights and degrees of deacetilation and, also, in the form of chitosan base or chitosan salt: e.g.. hydrochlorhydrate, glutamate, lactate).

Poly(oxyethylene) or poly(ethylene oxide) (PEO) is a synthetic non-ionic polymer. PEO and its block copolymers with poly(propylene oxide) (PPO) are available in the market with different molecular weights and various ratios of ethylene oxide to propylene oxide groups. These block copolymers, especially the one containing 80% ethylene oxide, have been extensively used in the preparation of parenteral colloidal drug carriers because of their lack of toxicity.

Bioactive macromolecules can be associated with these nanoparticles to different extents depending on the composition of the nanoparticles (on the ratio of the two main hydrophilic polymers and on the physicochemical characteristics of the macromolecule which is associated).

The incorporation of bioactive macromolecules within the nanoparticles can be achieved by a very simple and mild procedure which is particularly effective for preserving the stability of the macromolecules.

The formation of the nanoparticles occurs spontaneously due to the simultaneous precipitation of chitosan and the bioactive macromolecule caused by the incorporation of a molecule with a basic character, i.e. sodium tripolyphosphate (counter anion). This process can be also considered as a process of ionic gelation or ionic croslinking of chitosan with the counter anion. In this method, the utilization of organic solvents, extreme pH conditions or auxiliary substances of toxic nature are avoided.

The association of bioactive macromolecules with the nanoparticles occurs by a combined mechanism which may involve ionic and non-ionic interactions between the bioactive macromolecule and chitosan and a physical entrapment process. The ionic interaction between chitosan and negatively charged polymers has been previously described as the main mechanism involved in the formation of microcapsules by complex coacervation (T. Takahashi, K. Takayama, Y. Machida and N. Nagai, Chitosan-Alginate complex coacervate capsules: effects of calcium chloride, plasticizers and polyelectrolites on mechanical stability, Biotechnology Progress, 4, 76-81, 1988) and of polyion complexes (M.M. Daly and D. Knoor, Characteristics of polyion complexes of chitosan with sodium alginate and sodium polyacrylate, Int. J. Pharm. 61, 35-41, 1990). However, the association of bioactive macromolecules to nanoparticles made of chitosan or chitosan-PEO, according to an ionic interaction mechanism, has not yet been described. In addition, the originality here relies in the fact that the incorporation of the bioactive macromolecule into the nanoparticles occurs upon the incorporation of an ionic crosslinking agent such as sodium tripolyphosphate.

The current interest of hydrophilic nanoparticles is clearly illustrated by the growing amount of literature in this field. In this respect, it is worthwhile to mention several papers describing various methods of preparation of nanoparticles made of natural hydrophilic polymers and macromolecules (W. Lin, A.G.A. Coombes, M.C. Garnett, M.C. Davies, E. Stacht, S.S. Davis and L. Illum., Preparation of sterically stabilized human serum albumin nanospheres using a novel dextrano-MPEG crosslinking agent, Pharm. Res., 11, 1588-1592, 1994), (H.J. Watzke and C. Dieschbourg, Novel silica-biopolymer nanocomposites: the silica sol-gel process in biopolymer organogels , Adv. Colloid. Interface Sci., 50, 1-14, 1994), (M. Rajaonarivony, C. Vauthier, G. Courrage, F. Puisiex and P. Couvreur, Development of a new drug carrier from alginate, J. Pharm. Sci., 82, 912-917, 1993). However, the application of these nanoparticles for the association and delivery of high molecular weight active compounds such as peptides, proteins, antigens and oligonucleotides has not been described thus far. This could be partially due to the fact that most of the procedures described until now for the preparation of nanoparticles involve the use of organic solvents and/or covalent crosslinking agents as well as drastic conditions such as high temperatures or emulsification processes, which are extremely harmful for bioactive macromolecules. On the other hand, it has recently also been proposed the use of amphiphilic synthetic nanoparticles made of copolymers of lactic acid and PEO, for the delivery of macromolecules (P. Quellec, R. Gref P. Calvo, M.J. Alonso and E. Dellacherie, Encapsulation of a model protein and a hydrophobic drug into long-circulating biodegradable nanospheres, Proceed. Intern. Symp. Control. Rel. Bioact. Mater., 23, 815-816 1996). Once again, however, the main limitation of these nanoparticles is the necessity of using organic solvents and emulsification processes for their preparation.

Despite the important efforts which have been dedicated over the last years to the formulation of macromolecules, nothing has been reported so far dealing with the application of chitosan or chitosan-PEO nanoparticles for the associ-

ation and delivery of bioactive macromolecules with therapeutic or immunological interest. The preparation of chitosan nanoparticles without using harmful crosslinking agents such as aldehydes has not been yet reported either.

The new pharmaceutical composition described in this patent, based on the association of bioactive macromolecules to hydrophilic nanoparticles, overcomes problems previously encountered in the formulation of macromolecules. As indicated before, the main ingredients of the nanoparticles are two hydrophilic polymers: chitosan or chitosan salts and PEO or the block copolymers of poly(oxyethylene)-poly(oxypropylene) (PEO-PPO). The presence of PEO or PEO-PPO is not a requisite for the formation of the nanoparticles; however, the incorporation of these polymers in the system makes it more versatile since they affect the physicochemical properties of the nanoparticles such as the particles's size and zeta potential, as well as their release behavior and increase their biocompatibility. The chitosan: PEO ratio can vary enormously, reaching a value of 1:50. The association efficiency of the bioactive macromolecules to the nanoparticles can reach values as high as 100%.

The nanoparticles covered in this invention, which are intended for the association and delivery of bioactive macromolecules, offer numerous advantages over other types of nanoparticles previously described in the literature. These advantages rely not only in their preparation conditions but also from the point of view of their application for the administration of macromolecules by various routes. The most important benefits include: (1) the procedure for the incorporation of the bioactive macromolecule to the nanoparticles is instantaneous and does not require the use of ingredients which could be toxic for humans such as organic solvents, oils and aldehydic crosslinking agents; (2) the physicochemical properties of the nanoparticles, more specifically, their size, hydrophilic surface and surface charge, can be modulated by simply adjusting the ratio of CS and PEO; (3) these nanoparticles have an extraordinary capacity for the association of active ingredients of high molecular weight (macromolecules) and (4) they can deliver the associated active ingredient at different rates.

With respect to the routes by which these new nanoparticles can be administered to the human organism, it is convenient to distinguish between those which involve the contact of the nanoparticles with an epithelial or mucosal surface such as the buccal, oral, topical, transdermal, nasal, pulmonar, ocular and vaginal routes, and the parenteral routes which involve the injection of the coloidal particles. In the first case (epithelial, mucosal routes), the contact of the particles with the epithelium or the mucosa which are negatively charged can be favored by providing the nanoparticles with a high positive surface charge. In the second case (parenteral routes), especially following intravenous administration, these nanoparticles offer the possibility of modulating the biodistribution of the active molecules associated with them.

The nanoparticles covered in this invention are presented as colloidal suspensions in an external aqueous medium in which other ingredients i.e., cryprotective preservatives, viscosizers, salts..., could eventually be incorporated.

In the context of the present invention, the active ingredient (synonymous with a bioactive macromolecule) is the ingredient for which the formulation is designed and, therefore, the ingredient that will have a particular effect following its administration to an organism. The effect could be to prevent, palliate or treat a disease and also to improve the physical appearance (delivery of cosmetic agents...).

The pharmaceutical systems described here are characterized in that they have a size smaller than 1 μm (nanoparticles) and a great capacity for the association of bioactive macromolecules. The size of the nanoparticles is mainly dependent on the chitosan concentration in the nanoparticles formation medium. Thus, for a very low chitosan aqueous concentration (lower than 0.01%) or a very high chitosan aqueous concentration (higher than 0.5%), an aqueous gel solution or a suspension of microparticles (larger than 1 μm) is formed respectively. In addition, the size of the particles can be also modulated by incorporating PEO or PEO-PPO in the nanoparticles formation medium. As an example, results presented in Table 1 show the important augumentation in the nanoparticle size (from 275 nm to 685 nm) caused by the incorporation of increasing amounts of PEO-PPE in the medium (the chitosan/PEO-PPO ratio varied from 1/0 up to 1/50). Results in Table 1 also show that the incorporation of PEO-PPO to the nanoparticles led to a significant reduction in their zeta potential values.

The great capacity of the nanoparticles for the association of bioactive macromolecules has been demonstrated for several proteins (bovine serum albumin, insulin, tetanus toxoid, diphtheria toxoid) and oligonucleotides. Using bovine serum albumin (BSA) as a model therapeutic protein, it was shown that its association efficiency (percent of macromolecule incorporated with respect to the amount of macromolecule to be incorporated) to the nanoparticles was very high and influenced by the BSA concentration and the presence of PEO-PPO in the nanoparticles formation medium (Table 2). The size and zeta potential of the nanoparticles were not affected by the incorporation of BSA into the nanoparticles. On the other hand, it was observed that the stage at which the BSA was incorporated into the process has a remarkable effect on its association efficiency to the nanoparticles. Results in Table 3 reveal that a maximum association efficiency was achieved when the BSA was dissolved in the sodium tripolyphosphate aqueous solution and then added to the chitosan aqueous solution. In contrast, a minimum incorporation efficiency was obtained when the BSA was incorporated after the sodium tripolyphosphate, in other words, once the nanoparticles were formed. Finally, it was also observed that the pH of the nanoparticles formation medium has an important role in the incorporation efficiency of the protein into the nanoparticles. Results in table 4 indicate that the higher the pH, the more important was the percentage of BSA

incorporated into the nanoparticles. Results of the incorporation of tetanus and diphtheria toxoids into chitosan nanoparticles are presented in Table 5. These data provide evidence that the toxoids can be efficiently incorporated into the chitosan nanoparticles.

Another interesting feature of the chitosan nanoparticles described here is that they can deliver the macromolecule incorporated into them for extended periods of time. Furthermore, it was found that it is possible to modulate the release of the active ingredient from the nanoparticles by adjusting its loading and also by the presence of PEO-PPO in the nanoparticles.

Figure 1 displays the percentages of BSA released "in vitro" from nanoparticles made of different chitosan/PEO-PPO ratios, 1/0 (▲), 1/5 (Φ) and 1/25 (O), following their incubation at 37°C for different time periods.

Figure 2 displays the percentages of BSA released "in vitro" from nanoparticles containing different BSA loadings (amount of BSA entrapped in 100 mg of nanoparticles), 41% (O), 25% (Φ) and 20% (▲), following their incubation at 37°C for different time periods.

Results depicted in figure 1 indicate that the presence of PEO-PPO in the nanoparticles significantly increases the BSA in vitro release rate. Results showed in figure 2 indicate that the higher the loading, the faster the release rate is.

In summary, this invention covers a new pharmaceutical composition which can be used for the delivery of bioactive macromolecules following their administration by different routes: topic, oral, nasal, pulmonary, vaginal, ocular, subcutaneous, intramuscular and intravenous.

Some examples of the composition and preparation of various formulations of nanoparticles are described below.

Example 1

Association of BSA (bovine serum albumin) to chitosan nanoparticles. The composition of the formulation (nanoparticles suspension) in % (w/w) was as follows:

Chitosan base	0.14 %
Sodium tripolyphosphate	0.02 %
BSA	0.014%
Water	up to 100%

Chitosan was dissolved at the concentration of 0.2% (w/v) in 25 ml of 0.05M acetic acid solution. The pH of the solution was adjusted to pH 5.0. Then, 5 mg of BSA was dissolved in the chitosan solution. Finally, 10 ml of a sodium tripolyphosphate aqueous solution (0.1%, w/v) were added to the chitosan aqueous solution containing the BSA and the system was maintained under magnetic stirring for 30 min, after the spontaneous formation of the nanoparticles.

The size, zeta potential and BSA association efficiency for this formulation were: 402 nm, 46 mV and 100% respectively.

Example 2

Association of BSA (bovine serum albumin) to chitosan/PEO-PPO (1/5) nanoparticles. The composition of the formulation in % (w/w) was as follows:

Chitosan base	0.14 %
PEO-PPO	0.70 %
Sodium tripolyphosphate	0.02 %
BSA	0.014%
Water	up to 100%

Nanoparticles were prepared as described in example 1 with the exception that PEO-PPO was dissolved in the chitosan solution prior to the incorporation of BSA and the pH of the chitosan solution was adjusted to pH 4.0.

The size, zeta potential and BSA association efficiency for this formulation were: 519 nm, 44 mV and 78.2% respectively.

Example 3

Association of BSA (bovine serum albumin) to chitosan/PEO-PPO (1/25) nanoparticles. The composition of the formulation in % (w/w) was as follows:

Chitosan base	0.14 %
PEO-PPO	3.50 %
Sodium tripolyphosphate	0.02 %
BSA	0.014%
Water	up to 100%

Nanoparticles were prepared as described in example 1 with the exception that PEO-PPO was dissolved at the concentration indicated above in the chitosan solution prior to the incorporation of BSA and the pH of the chitosan solution was adjusted to pH 4.

The size, zeta potential and BSA association efficiency for this formulation were: 741 nm, 34 mV and 45.9 %, respectively.

Example 4

Association of tetanus toxoid to chitosan nanoparticles. The composition of the formulation in % (w/w) was as follows:

Chitosan base	0.14 %
Tetanus toxoid	0.014 %
Sodium tripolyphosphate	0.02 %
Water	up to 100%

Nanoparticles were prepared as described in example 1 except for adding tetanus toxoid instead of BSA to the chitosan solution at the concentration indicated above.

The size, zeta potential and tetanus toxoid association efficiency for this formulation were: 245 nm, 35 mV and 53 %, respectively.

Example 5

Association of diphtheria toxoid to chitosan nanoparticles. The composition of the formulation in % (w/w) was as follows:

Chitosan base	0.14 %
Diphtheria toxoid	0.007 %
Sodium tripolyphosphate	0.02 %
Water	up to 100 %

Nanoparticles were prepared as described in example 1 but adding diphtheria toxoid instead of BSA to the chitosan solution at the concentration indicated above.

The size, the zeta potential and the tetanus toxoid association efficiency for this formulation were: 245 nm, 36 mV and 55 %, respectively.

Table 1

Mean values of particle size and zeta potential of nanoparticles composed of different chitosan/PEO-PPO ratios.		
Chitosan/PEO-PPO (w/w)	Size* (nm)	Zeta potential # (mV)
1/0	275 ± 17	44 ± 1
1/2.5	283 ± 11	41 ± 2
1/5	300 ± 14	40 ± 1
1/25	430 ± 20	28 ± 1
1/50	685 ± 27	18 ± 1

* Determined by Photon Correlation Spectroscopy
# Determined by Laser Doppler Anemometry

Table 2

Particle size, zeta potential and association efficiency of chitosan nanoparticles containing different chitosan/BSA ratios.			
Chitosan/BSA Association (w/w)	Size * (nm)	Zeta potential #	
		(mV)	efficiency (%)
10/1	402 ± 24	45 ± 1	80 ± 3
4/1	359 ± 35	45 ± 1	43 ± 3
2/1	375 ± 26	45 ± 1	26 ± 1
1/1	368 ± 72	46 ± 2	21 ± 2

* Determined by Photon Correlation Spectroscopy
# Determined by Laser Doppler Anemometry

Table 3

Association efficiency of bovine serum albumin (BSA) to chitosan nanoparticles as a function of the stage at which BSA was incorporated and the theoretical chitosan/BSA ratio.		
Chitosan/BSA (w/w)	BSA association efficiency (%)	
BSA + nanoparticles	BSA + chitosan	BSA + TPP
10/1	10.8 ± 3	80.4 ± 3.2 100 ± 1.2
2/1		26.8 ± 0.7 45.2 ± 3.9
1/1		21.6 ± 2.0 41.8 ± 2.0

Table 4

Association efficiency of bovine serum albumin (BSA) to chitosan nanoparticles as a function of the pH of the chitosan solution and the theoretical chitosan/BSA ratio.		
Chitosan/ BSA (w/w)	BSA association efficiency (%)	
pH 3	pH 4	pH 5
10/1	66.8 ± 7.2	80.4 ± 3.2 91.7 ± 3.6
2/1	25.7 ± 1.4	26.8 ± 0.7 39.1 ± 2.4
1/1	19.4 ± 3.6	21.6 ± 2.0 35.5 ± 5.1

Table 5

Association efficiency of tetanus and diphtheria toxoids to chitosan nanoparticles.		
Toxoid	Chitosan/Toxoid	% Association
Tetanus	1/0,06	56.7 ± 2.7
Diphtheria	1/0,12	53,3 ± 4.2
Diphtheria	1/0,12	55,1 ± 5.5

Claims

1. Application of nanoparticles made of hydrophilic polymers as pharmaceutical forms for the administration of bioactive macromolecules, characterized in that their main ingredients are an aminopolyssaccharide, a salt from the phosphate family, a poly(oxylethylene) derivative and an a high molecular weight bioactive ingredient.

2. Pharmaceutical compositions according to claim 1, characterized in that they are formed spontaneously, in an aqueous medium, by ionic crosslinking and subsequent precipitation of the aminopolyssaccharide.

3. Pharmaceutical compositions, according to claim 1, characterized in that the nanoparticles have a size less than 1 µm, a positive electrical charge and a high capacity for the incorporation of bioactive ingredients.

4. Pharmaceutical compositions, according to claim 1, characterized in that said aminopolyssaccharide is selected from the group of chitosan or its derivatives.

5. Pharmaceutical compositions, according to claim 1, characterized in that said salt is selected from the group of monophosphates, diphosphates and polyphosphates or their derivatives.

6. Pharmaceutical compositions, according to claim 1, characterized in that said poly(oxylethylene) derivative is selected from the group of the poly(oxyethylene) and the block copolymers of the ethylene oxide and propylene oxide.

7. Pharmaceutical compositions, according to claims 1 to 5, characterized in that said active ingredient is selected from the group of macromolecules composed of peptides, proteins, polysaccharides and polynucleotides, having a therapeutic or antigenic activity.

8. Pharmaceutical compositions, according to claim 7, characterized in that said active ingredient is tetanus toxoid.

9. Pharmaceutical compositions, according to claim 7, characterized in that said active ingredient is diphtheria toxoid.

10. Pharmaceutical compositions, according to claim 6, characterized in that said poly(oxylethylene) derivative is incorporated in a percentage with respect to the total weight of the composition comprised between 0 % and 60 % (w/w).

11. Pharmaceutical compositions, according to claim 7, characterized in that said active ingredient (bioactive macromolecule) is incorporated in a percentage, with respect to the total weight of the composition, comprised between 0 % and 66 % (w/w).

12. Pharmaceutical compositions, according to claims 1 to 11, characterized in that they include an absorption enhancer.

13. Pharmaceutical compositions, according to claims 1 to 12, characterized in that they are presented in a form that is appropriate for parenteral administration or for their administration by various mucosal routes such as ocular, oral, nasal, vaginal, pulmonar and topical administration, their size being in the submicron range and their electrical charge variable (from +0.5 mV to +50 mV) depending on their final application.

Figure 1

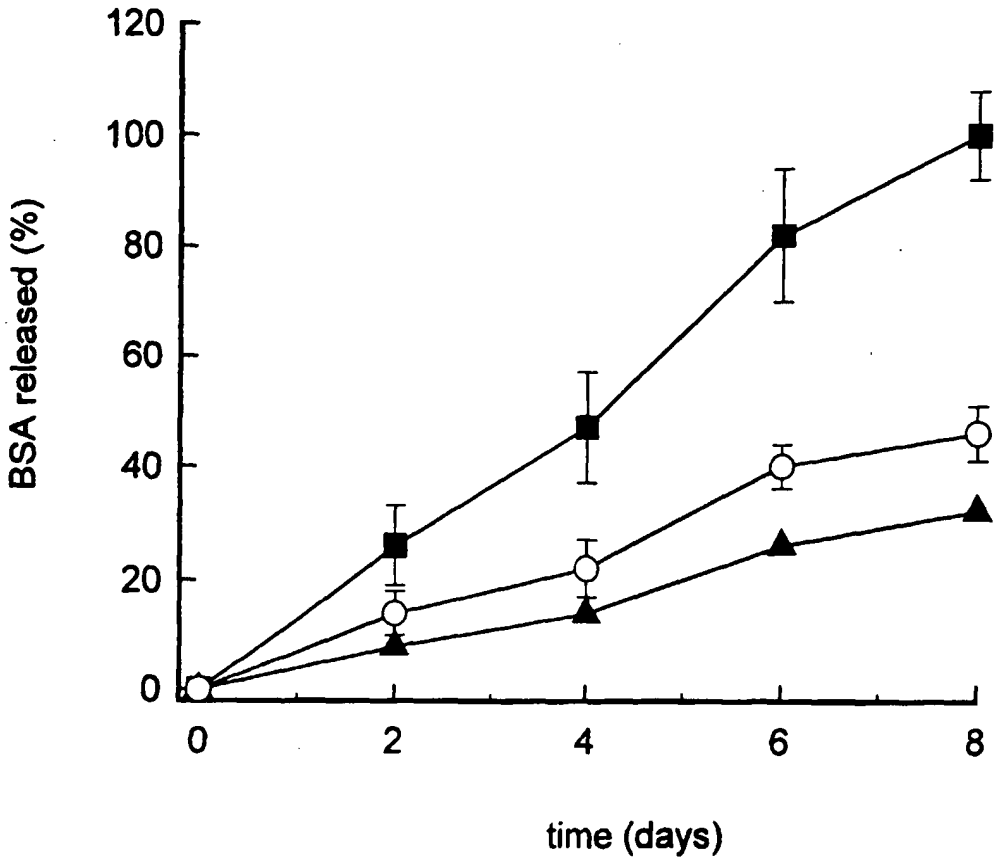
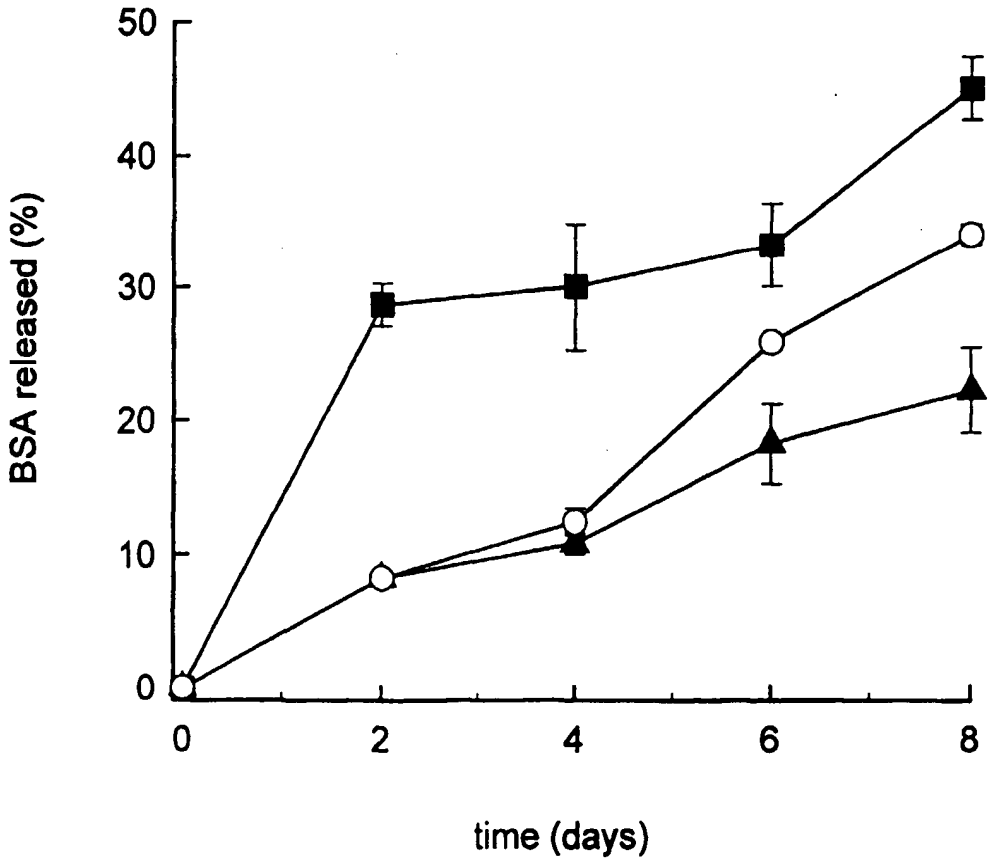


Figure 2



INTERNATIONAL SEARCH REPORT

International application No.
PCT/ES 96/00186

A. CLASSIFICATION OF SUBJECT MATTER

IPC[6]: A61K 9/51

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC[6]: A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5346703 A (T. X. VIEGAS ET AL.) 13 September 1994 (13.09.94) See the complete document	1-10
Y	WO 9605810 A (DANBIOSYST UK LIMITED) 29 February 1996 (29.02.96) See claims See page 4, line 20 - page 5, line 15 See page 11, line 9 - line 26	1-10
Y	WO 9620698 A (THE BOARD OF REGENTS OF THE UNIVERSITY OF MICHIGAN) 11 July 1996 (11.07.96) See claims 1-3, 10-12, 18-24 See page 7, line 1 - line 3 See page 11, line 2 See page 13, line 10 - line 14	1-10

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
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Development of adjuvant-active nonionic block copolymers

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Abstract

Nonionic block copolymers are surfactants synthesized using propylene oxide (PO) and ethylene oxide (EO) which are organized as 'blocks' of polyoxyethylene (POE) and polyoxypropylene (POP). These copolymers can be designed and synthesized using variable amounts of the PO and EO and with differential arrangement of the POP and POE blocks so that individual products have unique physicochemical properties. The copolymers that have been most thoroughly evaluated in vaccine research are linear with the polymer blocks organized as POE–POP–POE. Low molecular weight (MW) copolymers, 3–6 kDa, of this type have been used in oil-based emulsion formulations, whereas high-MW copolymers, >9 kDa, can be used in aqueous formulations. The adjuvant activity of nonionic block copolymers is influenced greatly by the size of the POP core block. As the size of this block is increased so is the adjuvant activity of the copolymer; peak activity is achieved using copolymers with POP cores that are 12–15 kDa. However, adjuvant activity is also affected by the amount of POE with low concentrations, 5–10%, being optimal. The type of immune responses produced is also influenced by the POE content. Copolymers with 10% POE preferentially augment Type 2 helper T-lymphocyte responses which support antibody responses, including mucosal antibody responses. Copolymers with <10% POE augment both Type 1 and Type 2 helper T-lymphocyte responses, which support a broader range of antibody responses and cellular immune responses. This property may allow for vaccines to be 'customized' by using adjuvant-active nonionic block copolymers that will augment the most appropriate types of immune responses. © 1998 Elsevier Science B.V.

Keywords: Adjuvant; Nonionic block copolymer; Parenteral vaccination; Oral vaccination; Emulsion; Cellular immunity; Antibody responses

Contents

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1. Introduction

Alkylene copolymers produced from EO and PO were developed specifically for use as surfactants [1,2]. The sizes and relative positions of the hydrophobic POP and hydrophilic POE blocks can be altered during synthesis by the order of monomer addition and amounts of monomer used and this, has allowed for the production of copolymers with significantly different surfactant characteristics. There are now more than 1000 documented excipient applications for alkylene copolymers, including their use as emulsifying, wetting, thickening stabilizing and dispersing agents in pharmaceutical and consumer products taken internally or externally. Thus, it has been possible to design 'customized' surface-active copolymers to suit a wide variety of applications.

The excipient uses of copolymers have been described in several detailed reviews and will not be covered herein [2–4]. Instead, we are providing information on the relatively new use of these copolymers as vaccine adjuvants. These new uses differ significantly from those for which this type of copolymer was developed and represent a new area with significant potential.

2. Molecular structures, synthesis and purification

2.1. Synthesis of copolymers

Alkylene oxide polymers are generally synthesized by the oxidation of alkenes and substituted alkenes. Common examples of alkylene oxides include ethylene oxide and propylene oxide which are produced mostly for the production of polymers and associated reaction intermediates. Polymers are produced through ring opening addition polymerization using a single alkylene monomer while copolymers are produced using two or more monomers, added in a predetermined sequence [2,5,6]. Copolymers of propylene oxide and ethylene oxide are produced by sequentially adding the monomers to active hydrogen atoms containing initiators in the presence of a suitable catalyst. Once the type and the amount of initiator is selected, the molecular weight of the POP base and weight percent of the POE units is determined by the amount of each monomer used. Several initiator molecules have been evaluated but propylene glycol is used most commonly. Similarly, numerous catalyst systems have been used but base catalyzed methods are the most widely accepted because of the simplicity and economics of the processes.

2.2. Nomenclature

Several names have been used to refer to alkylene oxide copolymers including polyalkylene oxides, polyoxyalkylenes, polyepoxides, polyols, poloxamers and polyoxiranes. Generally, homopolymers are referred by their monomer, such as polyoxyethylene or polyethylene oxide. When block copolymers are produced using more than one type of monomer, they are referred to by the respective monomers used and the order of arrangement. Thus, a triblock copolymer of ethylene oxide and propylene oxide

can be referred to as polyoxyethylene–polyoxypropylene–polyoxyethylene (POE–POP–POE) block copolymer.

The commercial manufacture of copolymers was pioneered by Wyandotte Chemicals Corporation and BASF Performance Chemicals (Parsippany, NJ) and the trade names that they developed, such as Pluronic®, Pluronic R®, and Tetronic®, Tetronic R® are still routinely used to refer to these different copolymers (Fig. 1). More descriptive terminology based on the relative composition of the POP and POE was also developed by BASF. An example of this system is shown for the Pluronic® copolymers and is called the Pluronic® grid (Fig. 2). The grid is formed by plotting the MW of the POP component against the amount of the POE, which is expressed as a percentage and is based on the MW of the intact POE–POP–POE product. The first two numerical digits are series numbers, which are related to the approximate MW of the POP base block as the last digit indicates the approximate percent of the POE. The prefix letter (L, P or F) denotes the physical form of the pure copolymer as liquid (L), paste (P) or flakes (F). For example, L121 and L122 are liquids, they have identical size POP blocks, with a MW of approximately 4000, and 10 and 20% of the intact copolymers are POE, respectively. The P123 and F127 copolymers are related because they are based on the same size POP block as the L121 and L122 but they contain 30 and 70% POE, respectively. The P123 is a paste, whereas the F127 is in flake form. Similar grids have been developed for Pluronic R®, Tetronic® and Tetronic R® copolymers.

The grid concept has been adapted further to simplify its use with nonproprietary materials. In the new system the MW of the POP and the percentage value of the POE are both divided by 10 and these values are substituted for the numbers used in the BASF name. Using the same example, the L121, L122 and P123 Pluronics® are referred to as Poloxamer 401, 402 and 403, respectively (Fig. 2).

Still other nomenclature has been developed for certain proprietary copolymers. Copolymers produced using modified manufacturing techniques by the CytRx Corporation (Norcross, GA) are referred to using an alpha-numerical designation that consists of the letters CRL, which stands for CytRx Research Laboratory, and four numbers. Unlike the poloxamer grid designations, the numbers are not based on physicochemical properties of the copolymer. In this review, we have generally referred to copolymers by the same names used in the cited papers. We have

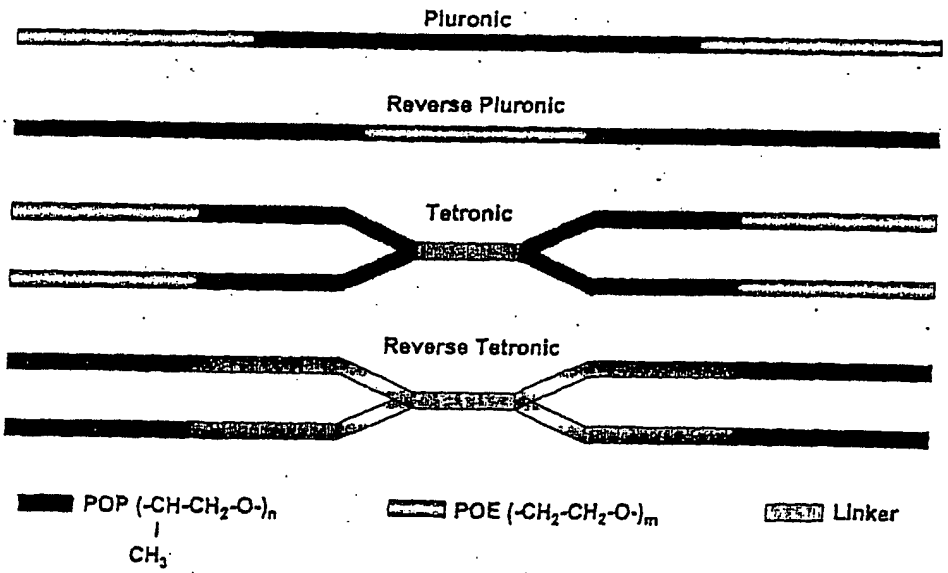

Fig. 1. Structures of nonionic block copolymers developed by BASF Performance Chemicals; the names Pluronic®, Pluronic R®, Tetronic®, and Tetronic R® are BASF trade names. The orientation of the polyoxyethylene (POE) and polyoxypropylene (POP) blocks can be varied, as shown. Both the orientation and size of the POP and POE blocks contribute to the unique surfactant properties of individual copolymer.

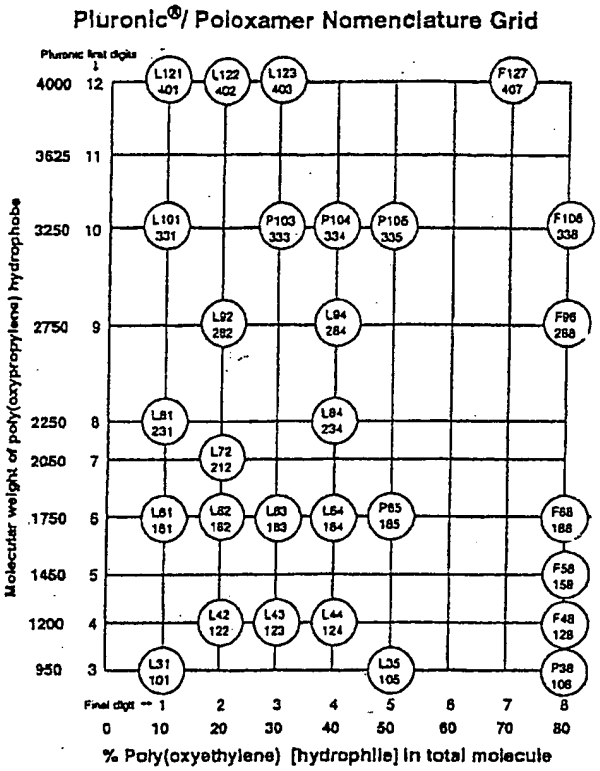

Fig. 2. The Pluronic® grid is formed by plotting the MW of the POP and percent of POE. In the grid developed by BASF for Pluronic® copolymers, the prefix letter (L, P or F) denotes their physical forms as liquid, paste or flakes, while the first two digits identify the series number which is related to the approximate molecular weight of the hydrophobic POP block. The last digit indicates the approximate percent of the POE, based on a ratio of POE:POP. A similar grid has been adapted to non-proprietary poloxamers by substituting the first two digits with actual molecular weight (in 100s) of the hydrophobic polyoxypropylene base block.

also provided added descriptions where we felt it was needed to avoid confusion, with respect to the identity of the copolymer product used.

2.3. Development of pharmaceutical grade copolymers

Quality specifications of poloxamers, POE–POP–POE copolymers, are often defined based on MW specifications, which means higher quality poloxamers will have a restricted MW distribution or reduced level of polydispersity [2,7]. Using this measure of quality, most commercially produced, industrial

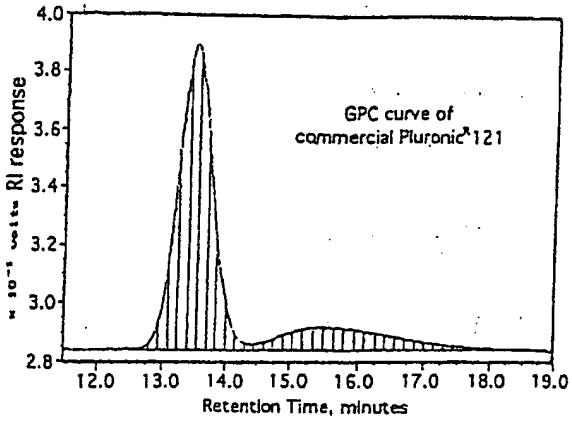

Fig. 3. GPC curve from analysis of Pluronic® copolymer L121. Data is reported as response versus retention time (min).

grade poloxamers are likely to be only adequate for surfactant uses because their MW distribution is too varied (Fig. 3). Thus manufacturing and purification methods that can produce more uniform poloxamers were needed if these materials were going to be acceptable for use in vaccines. The current generation of laboratory methodologies used to manufacture, purify and characterize copolymers are described in the following sections.

2.4. Analytical methods for the characterization of copolymers

2.4.1. Gel permeation chromatography

Gel permeation chromatography (GPC) is currently the most widely accepted and used method to characterize copolymers due to its simplicity and short assay time [7]. GPC is based on refractive index and is a 'relative method' since all measurements are compared to defined standards, usually propylene glycol (PEG). In GPC, it is assumed that the solution properties of the PEG and copolymers are similar which means this method provides only an estimate on the MW distribution, peak MW, weight average MW, number average MW and from these data the polydispersity can be calculated (Fig. 3).

2.4.2. Spectrometry based methods

Conventional mass spectrometric methods are not routinely used for copolymer characterization. How-

Table 1
Absorption peaks and chemical effects detected by infrared spectroscopy to identify specific functional groups in nonionic block copolymers

Absorption[a]	Functional Group	Mode
3500 cm^{-1}	Terminal Hydroxyl	O–H Stretching
2990 cm^{-1}	Methyl	Asymmetrical C–H stretching
2868 cm^{-1}	Methyl	Symmetrical C–H stretching
2368 cm^{-1}	C=O stretching	Atmospheric carbon dioxide
1480 cm^{-1}	Methylene and methyl	Scissoring of C–H bonds
1387 cm^{-1}	Methyl	Symmetrical C–H bending
1120 cm^{-1}	Ether Linkages	Asymmetrical C–O–C stretching

[a]IR absorption bands for various functional groups in nonionic block copolymers.

ever, techniques that are more accurate than GPC or that provide other types of information are likely to be needed for the manufacture of pharmaceutical grade copolymers and it is in this area that spectrometry based analytical methods may prove useful.

A technique, known as matrix-assisted laser desorption/ionization (MALDI) mass spectrometry, can been used to determine the same types of information obtained using GPC. In MALDI analyses, peptide standards of different MW are used instead of PEG, which is thought to provide more absolute MW measurements. However, the low-MW fractions of copolymer samples are more readily ionized and this needs to be considered in the analyses of data obtained from copolymer samples. Thus, this approach can be used to generate confirmatory data.

Infrared spectroscopy is not included in most analytical algorithms but it can be used to identify numerous chemical or functional groups. The functional groups, their absorption peaks and mode are summarized in Table 1. Analyses of these functional groups are likely to prove useful for quality control programs and as such, can be used to supplement GCP data.

Nuclear magnetic resonance spectroscopy (NMR) can be used to determine basic structural properties, such as the head-to-tail arrangements of the monomer blocks within copolymer molecules. For example, evidence for diblock rather than triblock structure is indicated by the presence of trace amounts of secondary hydroxyl groups in POE–POP–POE copolymer preparations. The technique can also be used to measure the weight percentage of POE blocks and levels of unsaturation. The expected chemical shifts for the protons in this type of copolymer are shown in Table 2.

2.5. Identification of copolymer by-products

Impurities present in block copolymers preparations can originate from catalyst, initiator, epoxide monomers and 'reaction by-products.' The reaction by-products are chemically related to intact copolymers and are the most common impurities [5,7,8]. These by-products are typically composed of EO

Table 2
Chemical shifts detected using NMR for protons in nonionic block copolymers

Proton detected[a]	Chemical shift (ppm)
–O-($CH_2CH(CH_3)$-O–; POP block CH_3	1.22–1.04
$HOCH(CH_3)CH_2$–; hydroxyl, broad, shift varies	2.62
–O-($CH(CH_3)$-CH_2-O), POP block	3.64–3.26
–OCH_2-CH_2-O–, POE block	3.73–3.62
$HOCH(CH_3)$-CH_2O–, secondary hydroxyl, end group	3.96–3.84
CH_2=CH-CH_2-O–, allyl unsaturation	4.00
CH_2=CH-CH-O–, allyl unsaturation	5.25–5.16
CH_2=CH–CH_2-O–, allyl unsaturation	5.90

[a]Effected protons are shown in bold.

and/or PO monomers that are incorrectly organized in copolymer molecules or low-MW copolymers with incorrectly proportioned POE and POP blocks. These by-products are formed when terminal unsaturation occurs during the polymerization of PO monomer units. Since this low-MW component originates during the hydrophobe POP formation, it is also present during the successive addition of EO. This is problematic because the more reactive EO monomers disproportionally react with the low-MW POP fractions and thus, the amount of EO added to the larger POP blocks is less than desired. These low-MW by-products appear as a separate peak in GPC analysis (Fig. 4). The amount of by-product impurities produced during synthesis increases with the size of the POP core. Thus, copolymers with hydrophobic POP core blocks greater than 4 kDa that are produced using standard manufacturing processes routinely have unacceptably high levels of reaction by-products.

Copolymer molecules with low-MW POP cores and only one flanking terminal POE block are also produced during synthesis. This occurs as the result of terminal unsaturation, which results in the development of either allyl ether or propenyl ether end groups. These unsaturation groups do not react with subsequently added EO monomer and copolymer by-products with only a single POE block are produced.

The presence of low-MW by-products with terminal unsaturation can severely affect the characteristics of the copolymer. Terminal unsaturation groups are more reactive and therefore more susceptible to oxidative reactions which reduces the stability of the copolymer. Other undesirable effects of terminal unsaturation may include the reduction of therapeutic potency of copolymers as well as increased toxicity caused by oxidative degradation products [6,8].

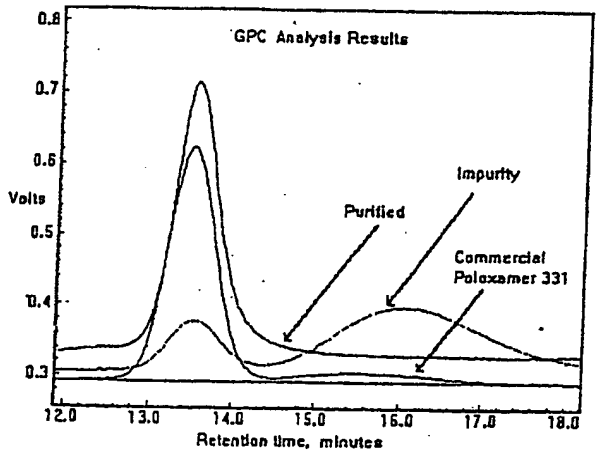

Fig. 4. GPC analyses of copolymer P331 prior to SCFE purification (commercial grade) and after purification. Analysis of purified reaction by-products is also shown (impurities).

2.6. Novel copolymer manufacturing and purification techniques

Numerous copolymer synthesis methods have been considered for the manufacture of POP-based block copolymers with the goal of reducing reaction by-product levels. These have included methods wherein reaction temperatures are lowered and/or reduced concentrations of monomer are used. New catalysts have also been evaluated, such as iron complexes, zinc complexes, double metal cyanide complexes, metallomorphines and alkali metals, such as cesium hydroxide [2,9]. These specialty catalysts have proved useful for the production of relatively high-MW copolymers with low unsaturation levels but they lead to the production of copolymers with an unacceptably high degree of polydispersity. For this reason, and their high cost, they are not economically attractive.

An alternative approach is to purify the desired fraction or to otherwise remove impurities using super critical fluid extraction (SCFE). This process is effective for removing low-MW by-products because the solvation properties of supercritical fluids can be accurately controlled to allow for the differentiation of copolymer products based solely on MW. We selected supercritical fluid carbon dioxide for this process primarily because the solvating power can be continuously varied by changing the pressure and temperature. An additional advantage of supercritical fluid carbon dioxide is the lack of residual solvents which limits potential toxicity and environmental impact concerns.

We developed this process using commercially available copolymers and, as shown by GPC analysis, low-MW by-products from poloxamer 331 (P331) can be effectively removed (Fig. 4). The MW distribution of the main peak does not change but the overall polydispersity of the product is significantly reduced. The advantages of purified copolymers

remains to be proved, but it is anticipated they will be more potent and stable since the reactive unsaturation groups are removed as a consequence of the removal of the low-MW by-products.

3. Use of copolymers to modulate immune responses

Compounds that modulate immune responses can function independently of a vaccine antigen or in conjunction with the antigen. Antigen-independent responses are also referred to as 'nonspecific' immune responses, whereas antigen-initiated or specific immune responses are often termed 'acquired' immune responses. Adjuvant augmentation of immune responses can involve both types of response types. However, the actual vaccine-induced response must always be antigen-specific. Nonionic block copolymers, particularly those with the POE:POP:POE block configuration characteristic of Pluronics®, have been carefully evaluated with respect to their adjuvant activity and we have provided information on both antigen-specific and nonspecific effects in the following sections.

3.1. Antigen-independent modulation of immune responses by Pluronic® copolymers

Compounds that nonspecifically activate the immune system generally function through direct or indirect activation of monocytes or macrophages. Examples include bacterial endotoxins, which directly activate macrophages, while products that induce inflammation at the site of injection, such as saponins and oil-based emulsions, indirectly activate macrophages as they respond to the injury. The production of both pro-inflammatory cytokines and inflammatory mediators nonspecifically activate B- and T-lymphocytes, which will then function in an antigen-specific manner.

Evidence from experiments in laboratory animals support the theory that the Pluronic® copolymers can directly activate macrophages. This was demonstrated using Pluronic® copolymer L81, which has a MW of approximately 3 kDa and is 90% POP and 10% POE. When this copolymer was administered intraperitoneally (i.p.) to mice in an oil-based emulsion, peritoneal macrophages were induced to express higher levels of Class II major histocompatibility (MHC) antigens [10]. Up-regulation of Class II MHC antigen expression is a standard surrogate measure of macrophage activation status. The macrophages from treated mice were primed for the production of superoxide anions and tumor-killing capabilities were also increased [10]. Thus, macrophage functions were also augmented by the treatment.

Phagocytic capabilities of macrophages can be used as an in vivo measure of their activation status [11,12]. In a mouse model, animals were given 15 mg/kg Pluronic® copolymer F68 by intravenous (i.v.) injection. Four days after the administration, mice were given an intravenous bolus of phagocyte resistant radioactive particles and the clearance of these particles from the peripheral circulation, which is mediated by macrophages in the liver, spleen and lymph nodes, was measured. In Pluronic® treated mice, almost 60% of the particles were cleared within 1 h, whereas only 32% of the particles were cleared in control treated animals. Clearance levels increased to approximately 76 and 53% for the treated and control animals after 5 h. The numbers of particles found in liver and spleen macrophages increased concomitantly, which suggests that the Pluronic® treatment increased macrophage phagocytic activity in vivo.

The mechanisms involved in the in vivo activation of macrophages by Pluronic® copolymers have not yet been defined but one likely possibility is that complement products are the mediators. The Pluronic® copolymers have been shown to activate the alternative complement pathway [13] and it is well known that certain complement proteins can activate macrophages and, thereby, induce an adjuvant effect [14,15]. Thus, the modulation of nonspecific immune responses may, in part, be the result of copolymer activation of the complement pathway rather than direct activation of macrophages.

3.2. Augmentation of antigen-dependent immune responses by Pluronic® copolymers

3.2.1. Use of emulsion-based vaccine formulations

Many adjuvant-active natural products are surface active and amphipathic in structure, similar to Pluronic® copolymers. Included are lipopolysac-

charides from Gram-negative bacteria, lipoteichoic acid from Gram-positive bacteria, trehalose dimycolate from mycobacteria, Quil-A and Qs-21 saponins from the bark of the soapbark tree, and a variety of natural surfactants [16–20]. Pluronic® copolymers with identical sized hydrophobic POP blocks, but different sized hydrophilic POE blocks, have been available commercially for many years. The ratio of POP:POE directly impacts the hydrophile–lipophile balance (HLB) of copolymers, and consequently, surface-active properties. This resource, therefore, provided the opportunity to directly investigate a possible correlation between surface activity and adjuvant activity.

The adjuvant activity of Pluronic® copolymers was originally demonstrated using low-MW Pluronic® copolymers, such as L101, P103 and F108 [21]. These copolymers are insoluble in aqueous buffers or saline and oils, so they were evaluated in oil-in-water and water-in-oil emulsions where the copolymer molecules are thought to align at the interface between the oil and water phases. The earliest studies were done using mice and demonstrated the potent adjuvant activity of Pluronic® copolymers with low HLB values. For example, hydrophobic copolymers such as L101 and L121, which are composed of POP and POE at a 10:1 ratio and have HLB values of 0.5 and 1, respectively, proved to be most active (Table 3). An optimal size of approximately 4 kDa was also observed which suggests the need for copolymers to be large enough, and thereby flexible enough, to allow them to interact with both the water and oil interfaces of the emulsions [22].

The identification of adjuvant-active copolymers led to the development of an adjuvant for use in research animals, termed TiterMax®. This product is a water-in-oil emulsion-based on squalene, which is a metabolizable oil derived from shark liver and certain plant oils [23]. The copolymer used is termed CRL8941, which has a MW of approximately 6000 and is 90% POP and 10% POE. The advantages of these materials, compared to the mineral oils and surfactants used in other water-in-oil emulsions, is their lack of toxicity.

The potency of TiterMax® adjuvant has been compared to other research adjuvants in controlled studies involving mice, rabbits and goats [24]. In these studies, the TiterMax® adjuvant augmented antibody responses greater than any of the other adjuvants including the complete and incomplete Freund's CFA/IFA water-in-oil emulsions without the site-of-injection toxicities commonly associated with the CFA/IFA. The utility of TiterMax® for inducing cytotoxic T-lymphocyte (CTL) responses has also been demonstrated. Immunization of mice

Table 3
Effects of HLB, size and POE content of nonionic block copolymers on adjuvant activity

Copolymer Identity[a]	HLB[b]	Molecular weight	Percent POE	Antibody Titer[c]	Percent Retention[d]
L31	1.0	1100	10	> 100	0
L81	2.0	2750	10	2178	ND[e]
L92	5.5	3650	20	1664	ND
L101	1.0	3800	10	84 338	70
L121	0.5	4400	10	67 814	99
L122	3.5	5000	20	184	ND
P103	9.0	4950	30	> 100	0
F108	> 24	14 600	80	> 100	0
None	—	—	—	> 100	0

[a]BASF designations.
[b]Hydrophile–lipophile value.
[c]Serum antibody levels to bovine serum BSA were determined by enzyme-linked immunosorbent assay (ELISA) using serum collected 4–6 weeks after a single, foot-pad immunization of ICR mice with 50 μg of BSA in an oil-in-water emulsion supplemented with 2.5 mg/dose of the selected copolymer.
[d]Retention in the foot pads was determined 7 days after immunization with 50 μg BSA in copolymer-supplemented oil-in-water emulsions. The equivalent of 25 μg BSA was administered into the foot pads in 50 μl of the emulsions and percent retention and was calculated based on the weight of emulsion recovered on day 7.
[e]Not determined.

with synthetic peptides containing known CTL epitopes reproducibly induced CTL responses while peptide alone or peptide formulated with alum failed to do so [25–27]. Thus, copolymer-supplemented emulsions appear to be adjuvant-active and capable of augmenting both antibody responses and cellular immune responses in many animals species.

Pluronic® types of copolymers can also be used in oil-in-water emulsions. Several groups have independently developed oil-in-water emulsion types of adjuvant products including Syntex Research (Palo Alto, CA) and IDEC Pharmaceuticals Corp. (San Diego, CA). The Syntex products are referred to as Syntex Adjuvant Formulations (SAF) and are composed of 5% squalane, threonyl-MDP, polysorbate-80 and Pluronic® L121 copolymer. [16,23,28]. The IDEC product is similar and composed of 15% squalane with polysorbate-80 and Pluronic® L121 and is referred to as antigen formulation (AF) or Provax® [23,29].

Both products are routinely microfluidized and consist of oil droplets with mean diameters of 150–175 nm. The emulsions are generally prepared without antigen, which can be added immediately before their administration. Thus, the proteins are not subjected to the shearing forces used to prepare emulsions and it is assumed they are more likely to retain native structure. This property, along with the ease of formulating vaccines, are advantages of the oil-in-water emulsions.

The SAF product has been tested in several species of research animals with a focus on its effects on antibody responses. This formulation significantly augmented antibody responses in mice, rats and guinea pigs and induced antibodies specific for native structure, supporting the theory that the emulsion did not denature proteins [30,31]. Protective immune responses to equine infectious anemia virus (EIAV) and simian immunodeficiency virus (SIV) have been induced in ponies and rhesus macaques, respectively, using experimental vaccines based on viral proteins and the SAF product [32–34]. Experiments using the AF formulation have focused on the induction of CTL responses. The AF product has proved to be a potent inducer of CTL responses in mice, including responses that protected animals from tumor challenge [29,35]. Neither of these formulations have been evaluated in human clinical trials but the results obtained from animal studies clearly support their evaluation for certain uses, such as cancer immunotherapy.

3.2.2. Mechanisms of action for Pluronic® copolymers in emulsions

As described in a previous section, emulsions containing the Pluronic® copolymers can activate macrophages via the alternative complement pathway [13]. However, this is probably only one of the ways that Pluronic® copolymers contribute to adjuvant activity. The Pluronic® copolymers also appear to stabilize water-in-oil emulsions and facilitate the binding of protein antigens at the interface between the oil and water phases [36]. Similarly, the oil droplets of the SAF and AF emulsions are thought to be coated with the Pluronic® L121 which should stabilize the interaction between protein antigens and the oil droplets. A model of the proposed interaction between Pluronic® copolymers, the hydrophobic oil and aqueous phases and vaccine antigens in emulsion formulations, is shown in Fig. 5.

Emulsions containing certain Pluronic® copolymers slow the clearance of antigens from the site of injection. Antigens injected into the foot pads of mice were found to be almost totally retained at that site when either L101 or L121 Pluronic® copolymers were included in formulation, whereas formulations containing P103 and F108 failed to alter clearance. The delayed rate of clearance was positively correlated with the magnitude of the antibody responses (Table 3) [21]. Thus, the adjuvant-active Pluronic® copolymers appear to have a depoting effect in vivo, at least when formulated in oil-based emulsions.

3.2.3. Potential medical utility for Pluronic® copolymers in emulsions

Water-in-oil emulsions are generally believed to be unacceptable for widespread use in human vaccines because of site-of-injection problems [37–41]. However, the use of emulsion components that are nontoxic, such as the metabolizable oils and pharmaceutical grade copolymers, or the use of oil-in-water emulsions, with $\leq 15\%$, oil may prove to be more acceptable. For example, cancer immunotherapeutic vaccines based on synthetic peptides containing CTL epitopes and administered in emulsions have proved

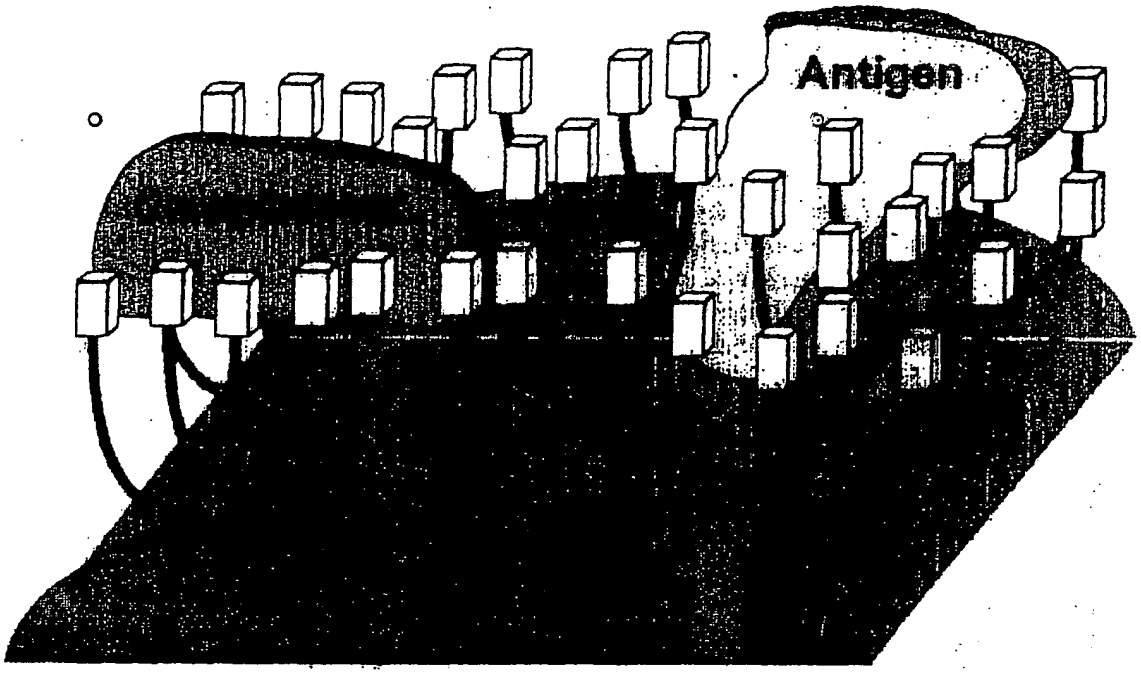

Fig. 5. Model of the proposed interactions between adjuvant-active copolymers, vaccine antigen and hydrophobic surfaces, which represent the oil phase in oil-in-water or water-in-oil emulsions. Copolymers are thought to align at the interface of the oil and water phases and antigens to interact or bind with both the POP and POE blocks.

immunogenic in animal models and similar products are being tested, or will soon be tested, in clinical trials [42–44]. In this setting, where the benefit:risk ratio is high, low levels of toxicity associated with the use of emulsions may prove acceptable.

3.3. Development of high-MW copolymers for use as vaccine adjuvants

3.3.1. Development of copolymers compatible with aqueous formulations

We have sought alternatives to emulsion formulations and this led to our current research focus on high-MW nonionic block copolymers. These 'next generation' copolymers are similar to the Pluronic® copolymers in orientation of the POE and POP blocks. However, they are significantly larger, containing 9–20-kDa POP cores flanked by small POE blocks. Like the Pluronic® copolymers, they are insoluble in aqueous formulations at elevated temperatures but can be solubilized at temperatures below their cloud points, which is about 8°C for most of these copolymers. As the temperature increases through the cloud point, the copolymer molecules aggregate into uniform sized detergent micelles or microparticles (Fig. 6). In this form, it is assumed that the hydrophobic POP portion of each molecule is positioned internally and that the hydrophilic POE component is exposed to the external aqueous environment. The low-MW Pluronic® copolymers can also be solubilized at low temperatures in aqueous buffers, but upon warming, they aggregate into strands or gels, which are not compatible with parenteral vaccine delivery [22].

The assembly of high-MW copolymers into micelles is influenced not only by the size of the molecule core but also the relative ratio of POP:POE blocks. Particle analyses using a series of related copolymers with an 11-kDa core but variable amounts of POE demonstrated that copolymers with 2.5–7.5% POE readily assembled into micelles in the 400 nm–3 μm size range (Fig. 7). As the solubility, or HLB, of the copolymer was increased, the size of the micelles decreased.

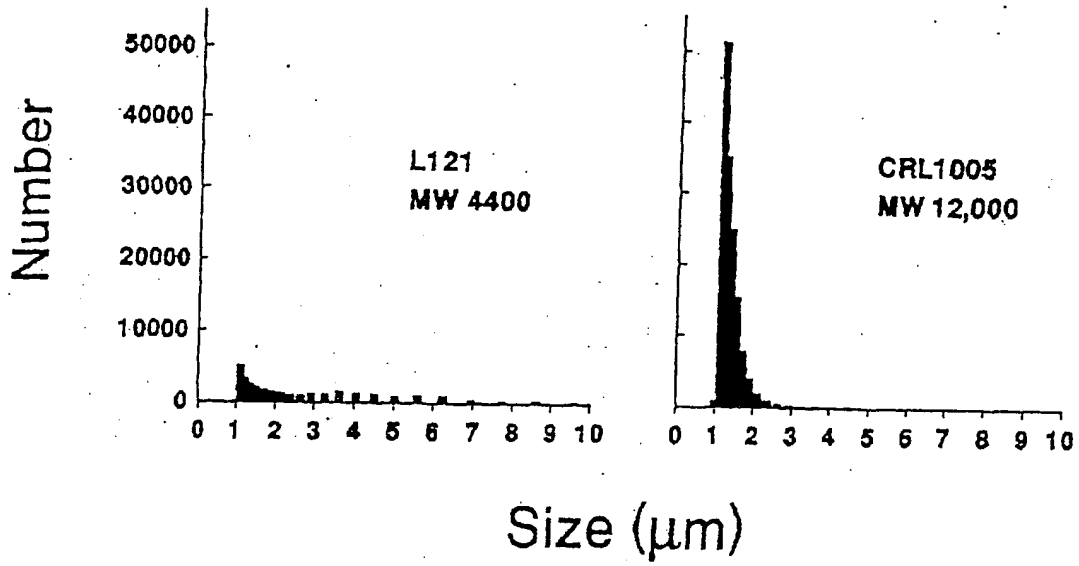

Fig. 6. The low-MW Pluronic® L121 copolymer and high-MW CRL1005 copolymer were cold solubilized at the concentration of 25 mg/ml in saline. Copolymer preparations were allowed to warm to room temperature and then analyzed using an Accusizer 770 particle sizer (Particle Sizing Systems, Inc., Santa Barbara, CA) which directly measures the numbers and sizes of particles by single particle optical sensing based on light obscuration. This instrument can accurately measure particles ≥ 1 μm, and the data are reported as numbers of particles with a given size in μm and data are expressed as particle number for each size channel in the range of 1–10 μm.

3.3.2. Adjuvant effects of high-MW copolymers

The adjuvant activity of the high-MW copolymers was demonstrated using a number of antigens including an influenza virus vaccine (Fluogen®, Parke-Davis, Morris Plains, NJ). This vaccine is a trivalent, 'split' virus preparation of which one of the predominant proteins is the influenza virus hemagglutinin (HA). The series of related copolymers with a POP core of 11 kDa were evaluated first. Formulations were prepared using a simple two-step process wherein the copolymers were mixed with the Fluogen® at room temperature and then chilled on ice to solubilize the copolymers. Formulations were then allowed to warm to room temperature so that copolymer micelles reformed. Antibody responses in BALB/c mice were measured 28 days after a single immunization. Copolymers with 2.5 and 5% POE were found to be potent adjuvants but the level of activity decreased when copolymers with higher POE concentrations were used (Fig. 10). These data support the observation made with the small Pluronic® copolymers, that copolymers with lower percentages of POE were more potent.

A second set of experimental vaccinations was done to identify the optimum size for the POP block. In this experiment, a second set of related copolymers with POP cores of 9–15 kDa, but all with 5% POE, were evaluated. Again, antibody responses of BALB/c mice were measured 28 days after a single immunization. Adjuvant potency increased as the size of the copolymer increased to the MW of 12 kDa but potency decreased at higher MW (Fig. 8). These data were used to identify a copolymer which we believe is optimal for use with the Fluogen® vaccine, having a POP core MW of 12 kDa and 5% POE. This copolymer is referred to as CRL1005.

Antibodies specific for the HA protein with the ability to block attachment and subsequent infection of influenza viruses, a process referred to as hemagluttination inhibition (HAI) [45], are critical for vaccine efficacy. The HA protein can be denatured in certain formulations, such as some emulsions, and this decreases the potency of the vaccine, at least with respect to the production of antibodies with HAI activity. Evaluation of sera from mice immunized with the Fluogen® vaccine supplemented with CRL1005 demonstrated that HAI titers were increased similar to total titers. The CRL1005 co-

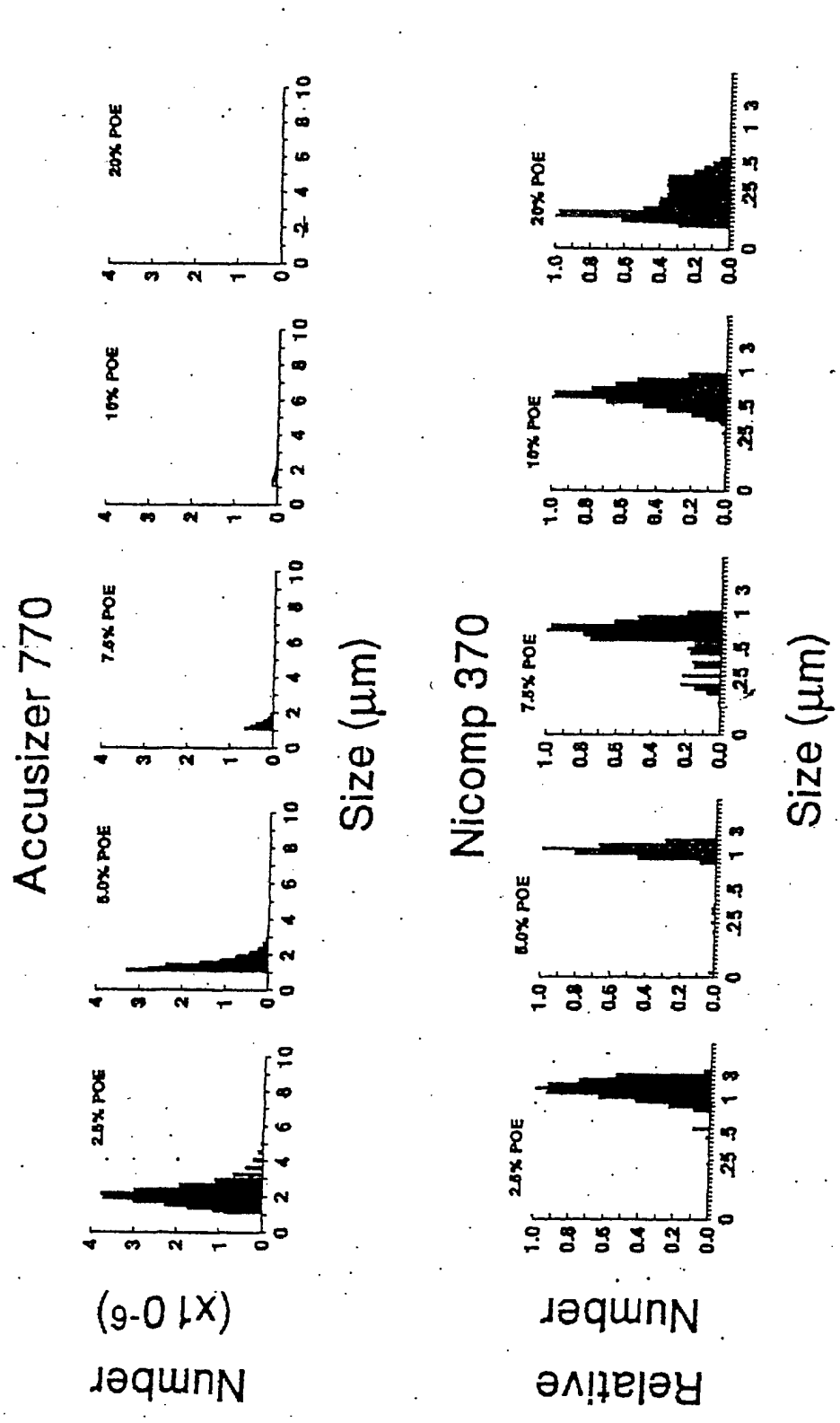

Fig. 7. Particle size analyses of a series of nonionic copolymers with 11-kDa POP cores and variably sized POE blocks of 2.5, 5.0, 7.5, 10 and 20%. Copolymers were cold solubilized at the concentration of 25 mg/ml then allowed to warm to room temperature prior to analyses using the Accusizer 770 (upper) and Nicomp 370 (lower) particle sizers (Particle Sizing Systems, Inc., Santa Barbara, CA). The Accusizer 770 directly measures the numbers and sizes of particles ≥ 1 μm by single particle optical sensing based on light obscuration and data are expressed as particle number for each size channel in the range of 1–10 μm. The Nicomp 370 measures the size distribution of particles using dynamic light scatter at 90°. Because this instrument determines the relative, rather than absolute numbers of particles within a designated size range, the data are reported as relative number in each size channel in the range of 1 nm–3 μm with the channel containing the greatest number arbitrarily set to 1.0.

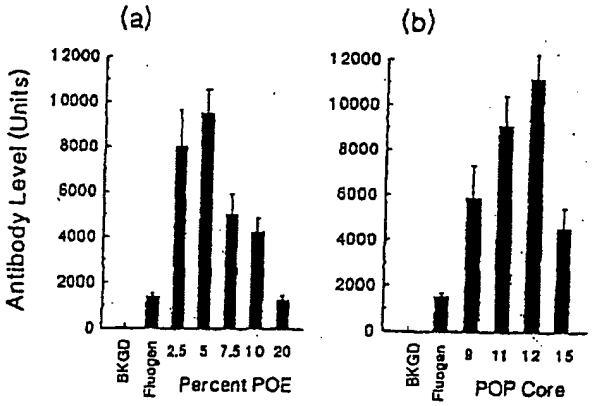

Fig. 8. Influence of POP and POE block size on adjuvant activity of high-MW copolymers was determined using a trivalent influenza virus vaccine (Fluogen®). This vaccine is a 'split' product and contains viral proteins from the A/Texas/36/91 (H1N1), A/Johannesburg/33/94 (H3N2) and B/Harbin/7/94. The experimental formulations were supplemented with nonionic block copolymers from the series based on the 11-kDa POP core and variable POE components (a). The second series contained copolymers with POP core blocks of MW 9, 11, 12 and 15 kDa, but all with 5% POE (b). Experimental formulations were prepared by mixing the Fluogen® vaccine, containing 90 μg/ml HA, with 50 mg/ml copolymer in PBS. Female BALB/c mice, 6–8 weeks old, 10 mice/group were immunized once at the base of the tail with 4.5 μg of Fluogen, based on hemagglutinin (HA) content, in a total volume of 100 μl, with or without 2.5 mg/dose of selected adjuvant-active copolymers. Serum samples were collected 28 days after a single immunization and the levels of serum antibodies specific for influenza virus proteins were determined by ELISA. Antibody levels were determined using a reference standard and are expressed as Units. Control vaccine formulations consisted of Fluogen® alone, and were used to determine responses to the standard vaccine. Immunization with saline was used to determine the background levels (BKGD).

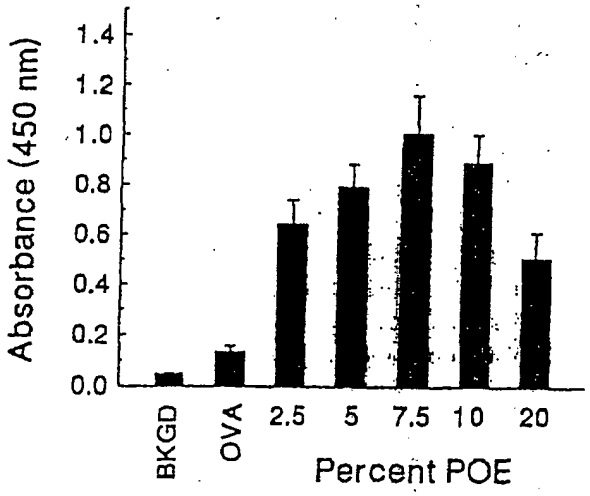

Fig. 9. Augmentation of antibody responses to OVA by block copolymers composed of an 11-kDa core and varying concentrations of POE (2.5–20%). Groups of 10 C57BL/6 female mice, 8 weeks old, were immunized s.c. twice at 28-day intervals with 25 mg/dose of OVA supplemented with 2.5 mg/dose of individual copolymers. Sera were collected on day 56 and levels of specific antibodies determined by ELISA. Control vaccine formulation was OVA alone and PBS was used as the vaccine to the background (BKGD) control. Data are shown as the mean absorbance (450 nm) obtained using sera diluted 1/100.

polymer appears to be ideally suited for viral protein-based vaccines, such as the Fluogen® vaccine.

One cannot assume that the CRL1005 copolymer will be optimal for use with other proteins, so a similar study was done using ovalbumin (OVA) as the experimental vaccine antigen and the same series of copolymers (Fig. 9). With the OVA, copolymers with the 11-kDa POP core and 7.5–10% POE were the most potent. Thus, matching of protein antigens with copolymers may be needed to identify the most potent combinations. Currently, optimal combinations are selected using animal immunogenicity experiments but it may be possible to make appropriate selection based on the physicochemical characteristics of the immunogen and copolymers.

The adjuvant activity of high-MW copolymers on cell-mediated immunity has been evaluated primarily using OVA focusing on the differential augmentation of T-helper type 1 (Th1) and T-helper type 2 (Th2) lymphocyte responses. Immunization studies were done using copolymer with a POP core of 11 kDa and 5 or 10% POE. Adjuvants used for comparative purposes were aluminum hydroxide (alum), which is known to augment Th2 responses, and saponin (Quil-A), which augments both Th1 and Th2 responses and induces cytotoxic T lymphocyte (CTL) responses [46]. Cellular immune responses were characterized as Th1 and/or Th2 based on the types and amounts of cytokines produced in vitro by lymphocytes from immunized mice; interleukin-5 (IL-5) and IL-10 were used as indicators of Th2 responses while gamma-interferon (γ-IFN) and IL-2 were used as indicators of Th1 responses. The induction of cytotoxic T lymphocyte CTL responses, which is Th1 dependent, was also assessed.

Splenic lymphocytes from mice immunized with

formulations containing the control adjuvants produced the types of cytokines that were predicted. Alum augmented the production of only IL-10 and IL-5, characteristic of Th2 responses, whereas Quil-A augmented the production of γ-IFN and IL-2 as well as IL-5 and IL-10, indicating Th1 and Th2 responses (Table 4). The adjuvant activity of the two copolymers was different and correlated to the POE content. The more hydrophobic copolymer augmented mixed Th1 and Th2 responses, whereas the 10% POE copolymer augmented primarily IL-5 and IL-10, Th2 cytokines.

The different control and copolymer adjuvants also induced CTL responses in the expected pattern. Quil-A and copolymer with 5% POE were potent CTL adjuvants but, whereas the alum and 10% POE copolymer failed to induce comparable responses (Table 4). Based on these findings, we believe it may be possible to partially direct vaccine-induced immune responses towards the type of response that is needed by the use of a high-MW copolymer with the appropriate HLB.

3.3.3. Mechanisms of action for high-MW copolymers

If the data obtained using the OVA and Fluogen® are considered together, then it becomes obvious that adjuvant activity and the ability of the high-MW copolymers to assemble into particles are correlated with low HLB values. These findings are similar to those reported for the Pluronic® copolymers and it is therefore assumed that similar mechanisms are involved with adjuvant activity. These include formation of depots, increasing uptake of antigen by macrophages and activation of macrophages by copolymers. The difference is that the oil phase of the emulsion has been replaced by the POP portion of the high-MW copolymers within the micelles.

In initial experiments using the high-MW copolymers and the Fluogen®, formulations were prepared with and without the copolymer cold solubilization step. Formulations that were simply mixed and administered to mice were significantly less potent than those that were prepared with the cold solubilization step. This finding supports our theory that optimal adjuvant activity requires the vaccine immunogen and the copolymer to associate together into micelles. To test this theory, particle size analyses were used to characterize micelles of the CRL1005 copolymer when formulated alone or with either OVA or the Fluogen® vaccine. Micelles formed using CRL1005 alone were in the 500 nm–1 μm size range and appeared to be composed of smaller units that aggregated into the larger micelle

Table 4
Effects of adjuvants on the augmentation or induction of Th1 and Th2 lymphocyte responses[a]

Adjuvant used[b]	Th1 cytokine levels (pg/ml)		Th2 cytokine levels (pg/ml)		CTL responses (% release)	
	γ-IFN	IL-2	IL-5	IL-10	Test	Control
Alum	98	0	5554	5121	8	2
Quil-A	318	38	5011	6234	54	8
5% POE	327	48	6456	6497	39	7
10% POE	156	0	5134	4513	8	5

[a]Augmentation of OVA-specific Type 1 (Th1) and Type 2 (Th2) cellular immune responses by the use of adjuvants was assessed using antigen-driven cytokine production in vitro. C57BL/6 mice were immunized twice (days 0 and 28) with 25 μg/dose OVA formulated with different adjuvants. On day 56, splenic leukocytes were recovered and cultured for 5 days using standard techniques with or without OVA (25 μg/ml) and the concentration of secreted cytokines in culture supernates was determined using a 'capture ELISA' based on a standard curve and commercially available antibodies (PharMingen, Los Angeles, CA). Data represent (cytokine concentration in OVA stimulated cultures) − (cytokine concentration in control cultures) and are expressed as pg/ml. CTL activity was measured using C57BL/6 mice following immunization with the same adjuvants and immunization schedule. Splenic lymphocytes were cultured with mitomycin-treated, OVA-transfected EG-7.OVA cells to induce maturation of precursor CTL. The CTL activity was measured using a standard release assay and the EG-7.OVA or the OVA-negative EL-4 parent cells as target cells. Data are shown as the percent specific release (test release − spontaneous release/maximum release − spontaneous release) × 100 obtained using EG-7.OVA target cells or control EL4 target cells and an effector:target cell ratio of 50:1.

[b]Adjuvants known to induce Th1 responses, Quil-A, and Th2 responses, alum, were used as controls and for comparison. Copolymers with 11-kDa dalton POP cores and 5 and 10% POE were used as the experimental adjuvants.

(Fig. 10). The formulations containing OVA or the Fluogen® vaccine were significantly different with respect to the size distributions of the micelles; they were smaller when formulated with the Fluogen® and larger when formulated with OVA. Thus change of micelle size provides direct evidence for physical interaction between the proteins and the copolymer.

Micelles of CRL1005 copolymer formulated alone or with these proteins have been visualized using scanning electron microscopy (SEM). Micelles of CRL1005 were found to be spherical but slightly irregular in shape and composed of smaller units aggregated into the larger micelles. Micelles composed of OVA and CRL1005 copolymer were similar in appearance but larger, whereas those containing the Fluogen® vaccine were smaller, more uniform and smoother in appearance [47,48]. Thus, the size and physical characteristics of the high-MW copolymer micelles are affected by the protein in the formulations. Also, the size characteristics of the micelles can be used to confirm interaction of the copolymer with the vaccine antigen.

The physicochemical mechanisms responsible for the interaction between proteins and the high-MW nonionic block copolymers are not known. The hypothesis developed for the Pluronic® copolymers is based on hydrogen bonding and this same mechanism could well be involved. We believe that proteins can interact through the hydrophilic POE block where hydrogen bond donor sites exist on the two terminal hydroxyl groups or through the hydrophobic POP block where ether-linked oxygens provide

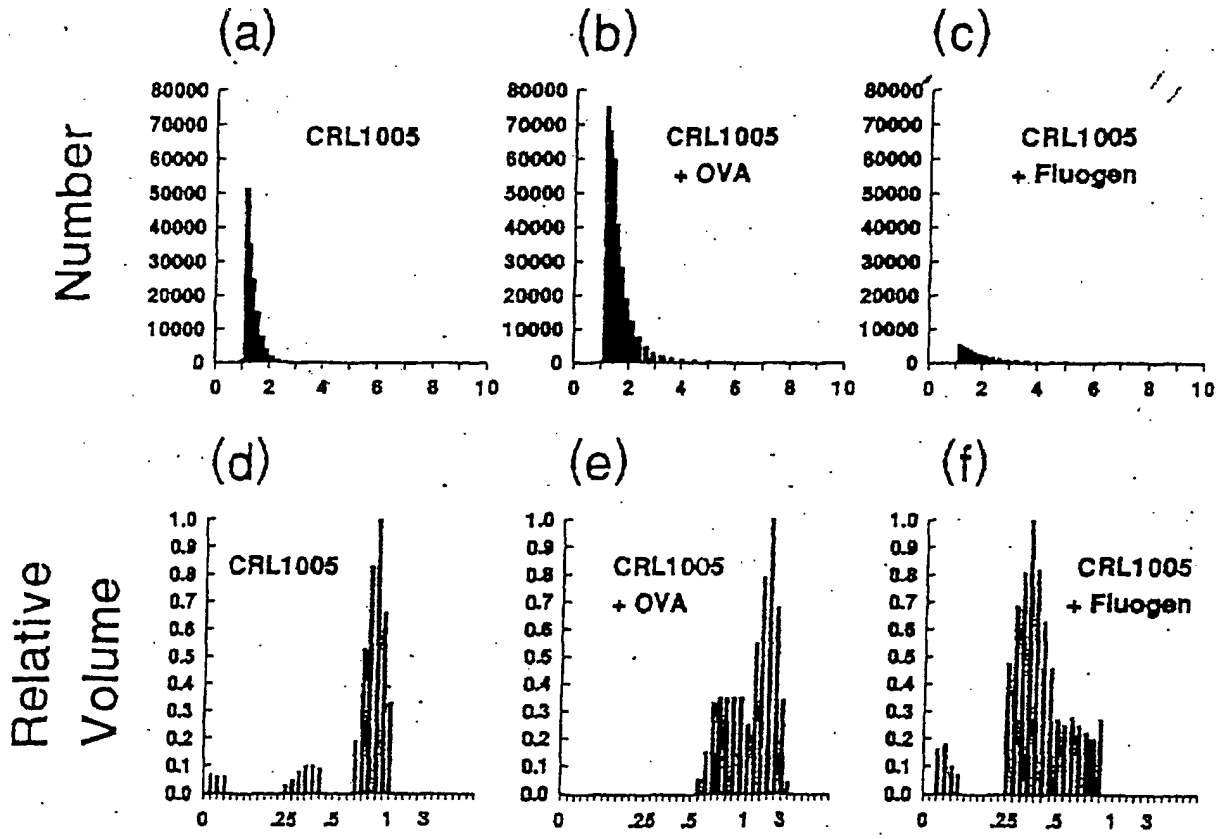

Fig. 10. Particle analyses of micelle formulations containing 25 mg/ml CRL1005 alone or mixed with 250 μg/ml OVA or 45 μg/ml Fluogen™. Particle analyses were done using the Accusizer 770 and Nicomp 370 particle sizers. The results of analyses using the Accusizer (upper) for CRL1005 alone (a), with OVA (b) or Fluogen™ (c) are expressed as number of particles per channel. 1–10 μm. Data obtained using the Nicomp (lower) are reported as relative number in each size channel in the range of 1 nm–3 μm with the channel containing the greatest number arbitrarily set to 1.0 (d,e,f).

multiple hydrogen bond acceptor sites. The points of interaction are probably dictated by the physicochemical characteristics of the protein. Soluble proteins, such as OVA, most likely associate with the terminal POE components of the copolymer molecules while proteins with large hydrophobic regions, such as transmembrane regions of HA, may interact with the hydrophobic POP component. Large complex proteins, such as HA which contain both hydrophobic and hydrophilic regions, most likely interact with both the POP and POE blocks and are therefore, ideally suited for formulation with this type of copolymer.

If it is accepted that the vaccine protein and copolymer must interact so that both are contained within the micelle for optimal adjuvant activity, then it is also logical to assume that no single copolymer will be optimal for use with all proteins. The studies completed using the series of copolymers with an 11-kDa POP core and OVA as the antigen support this idea. In these studies, the copolymers with 7.5–10% POE were more potent than the copolymer with 5% POE, which was optimal for the Fluogen® vaccine. The reason for this is unknown but we believe that the copolymer molecules with larger POE blocks are more likely to interact with highly soluble proteins, such as the OVA. Thus, copolymers with concentrations of POE $\leq 5\%$ appear to be optimal for use with proteins that contain hydrophobic regions, whereas highly soluble proteins are better matched with copolymers that have higher concentrations of POE.

The amount of POE incorporated into copolymers appears to have other effects relative to adjuvant activity. The studies completed using OVA demonstrated differential activities with respect to the induction or augmentation of cellular immune responses. The more hydrophilic copolymer augmented primarily Th2 types of responses, whereas the hydrophobic copolymer augmented both Th1 and Th2 responses. The reasons for this differential activity have not yet been elucidated but we believe the activity relates to HLB. Nonionic block copolymers are not lytic types of surfactants but are highly surface-active and it is possible that those with the lower HLB values can facilitate the nonspecific transfer of protein antigens into the cytoplasm of cells. This would lead to the induction of Th1 responses and CD8+ CTL, which were observed, in a manner similar to saponins, such as Quil-A and Qs-21 [49–53], or liposomes [54–56]. It appears likely that one can match copolymers with protein antigens to identify optimal of combinations to selectively drive the cellular responses.

4. Toxicity studies and clinical testing

Toxicity is one of the most significant hurdles in any vaccine adjuvant development program. For prophylactic vaccines designed for use in the general population, such as the Fluogen® vaccine, even increased rates of localized pain or swelling at the site of injection are unacceptable. Today, most of the experimental adjuvant-active compounds that have been developed to the point of testing in human clinical trials have recognizable toxic effects associated with their use.

Pluronic® copolymers are used as excipients in many pharmaceutical products and have proved nontoxic at the dose levels that would be used in vaccines. This historical database provides a certain level of confidence with respect to the use of copolymers in vaccines. However, the high-MW series of copolymers have not yet been tested thoroughly enough to document activity and safety. Our development program was narrowed to focus on a single copolymer, CRL1005, and included evaluation of adjuvant activity in nonhuman primates, and extensive toxicity testing in rabbits, guinea pigs and mice to establish the level of toxicity.

The CRL1005 copolymer was evaluated in combination with the Fluogen® vaccine using rhesus macaques. Since rhesus macaques are not readily infected by human influenza viruses, the animals were considered to be immunologically naive and two vaccinations were administered at 28-day intervals. Full 'human' doses were given, with or without 25 mg/dose of the CRL1005 copolymer and antibody responses were measured using serum and standard assays. Vaccination with the Fluogen® vaccine alone failed to induce detectable antibody responses until a second immunization was given, and even then the antibody levels were very low (Fig. 11a). The CRL1005 supplemented formulation

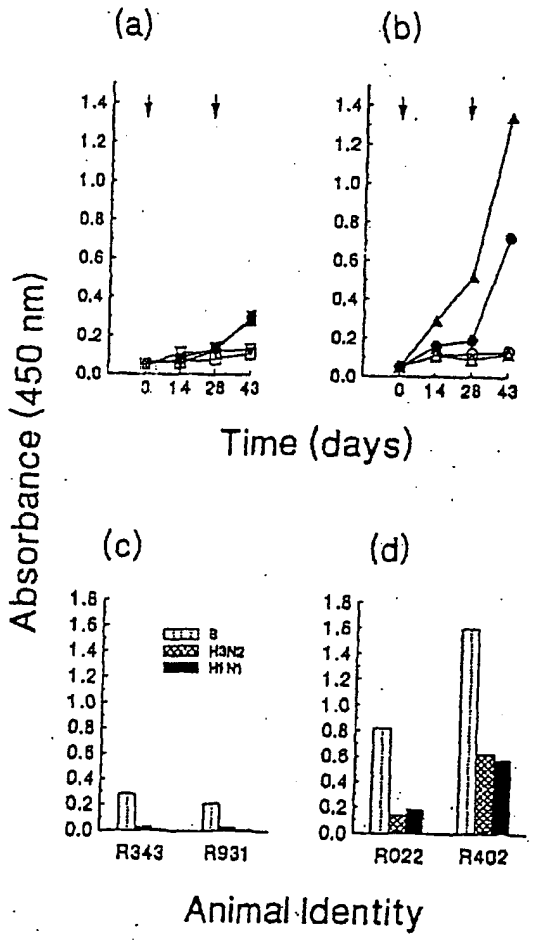

Fig. 11. The adjuvant activity of CRL1005 copolymer in rhesus macaques was determined using Fluogen® influenza virus vaccine. The experimental formulation contained 45 μg of Fluogen® and 25 mg of CRL1005. Fluogen® alone was used as the control formulation. Four male rhesus monkeys, 3–4 years old, were immunized intramuscularly, twice at 28-day intervals indicated with the arrows (↓). Serum samples were collected at 14–15-day intervals and the levels of serum antibodies specific for influenza virus proteins were determined by ELISA using both the Fluogen® vaccine (a,b) and individual influenza virus type A and B preparations (c,d). Kinetics of serum antibody responses induced in rhesus monkeys following two immunizations, on days 0 and 28, with Fluogen (45 μg HA) administered alone (a) to monkeys R343 (▼) and R931 (■) or supplemented with 25 mg of CRL1005 copolymer (b) to monkeys R022 (●) and R402 (▲). Antibody levels are shown as solid symbols when tested against the Fluogen® and as the open symbols when tested without an antigen. Data are expressed and the ELISA absorbance value (450 nm) obtained using a 1/100 dilution of serum. Antibody levels to individual variants were similarly determined using serum samples obtained on day 43 tested at a 1/100 dilution (c,d).

induced significantly higher antibody responses after each immunization (Fig. 11b). The effect of the CRL1005 was to augment antibody responses to all three of the virus types present in the Fluogen® vaccine, not just the immunodominant Type B variant (Fig. 11c,d) [47]. Detailed toxicity testing was not included in this experiment but routine physical examinations were done and site-of-injection toxicities were not observed. This experiment clearly demonstrated the adjuvant activity of the CRL1005 in primates and supported continued development of this product for potential use in human vaccines.

Prior to evaluation in a clinical trial, more extensive toxicity testing of the CRL1005 was completed. This included testing rabbits using a repeat immunization schedule and human doses, in mice and guinea pigs in the General Vaccine Safety Test (Table 4) and in rabbits to determine pyrogenicity (Table 5). The CRL1005 copolymer proved to be nontoxic in all three tests and was therefore considered safe for clinical evaluation.

The vaccine immunogen selected for use in the first clinical trial, CTP37-DT, is based on a synthetic peptide homologous for the carboxyl-terminal 37 amino acids (CTP37) of the β-chain of human chorionic gonadotropin (βhCG), which is conjugated to diphtheria toxoid (DT). This cancer vaccine was evaluated previously in humans in a water-in-oil emulsion containing MDP as the adjuvant, a product that is similar to CFA. This CFA-like emulsion augmented antibody responses but significant site-of-injection reactions were noted and cellular immune responses were not induced [40]. Thus, a new adjuvant that is capable of augmenting cellular immune responses with a more acceptable safety profile is clearly needed and this was the reason for evaluating the CRL1005 copolymer adjuvant.

The Phase I clinical trial was designed using a dose escalation of the CRL1005 copolymer, 3, 10, 25 and 75 mg/dose, and a single dose level of the CTP37-DT, 1 mg/dose [57]. The experimental vaccine formulations were well tolerated at all CRL1005 copolymer dose levels with no systemic toxicity reported and localized site-of-injection toxicity comparable to levels expected for standard vaccinations. Antibodies specific for βhCG were detected at some time during the study for all of the patients receiving

Table 5
Safety testing of CRL1005 copolymer

Type of Testing	Species and strain	Test Agent(s)	Dose (s) (mg/ml)[a]	Day of dosing[b]	Route of dosing	Results
CRL1005 dose escalation, multiple doses[c]	Rabbits, ♂ & ♀, New Zealand White	CRL1005 tested alone	12.5, 5, 25 and 75	0, 28 & 56	Intra-muscular	No systemic or local toxicity
General vaccine safety[d]	Mice, ♂, CD-1, (ICR)BR	CRL1005 + CTP37-DT[e]	3, 10, 25 and 75	0	Intra-peritoneal	Passed, no weight loss
General vaccine safety[d]	Guinea pigs, ♂, (HA)BR	CRL1005 + CTP37-DT[e]	3, 10, 25 and 75	0	Intra-peritoneal	Passed, no weight loss
Pyrogenicity[f]	Rabbits, New Zealand White	CRL1005 + CTP37-DT[g]	75[h]	0	Intra-venous	Passed, no fever induced

[a]Doses listed are for the CRL1005 copolymer only. The CTP37-DT was used at the concentration of 1 mg/ml. When CRL1005 copolymer and CTP37-DT were formulated together, the doses shown are final concentrations.
[b]Studies all started on day 0.
[c]Five groups of rabbits, consisting of four males and four females, were used for the study. Four groups were administered increasing amounts of CRL1005 while the fifth group received only phosphate-buffered saline (PBS) vehicle. Treatments consisted of injection of 1 ml CRL1005 in PBS into the the quadricepts. Injection sites were observed and body temperatures recorded at 0, 24, 48 und 96 h after each injection, and at weekly intervals throughout the study. Blood was collected 4 days after each injection for clinical chemistry and hematology analyses. Animals were sacrificed on days 84 or 85, a gross necropsy performed and selected tissues fixed for histopathological evaluation.
[d]The General Vaccine Safety Test was run in accordance with FDA guidelines (Code of Federal Regulations 21 610.11 (1993) 46–48.
[e]Only formulations of CRL1005 combined with CTP37-DT were tested. Mice were administered 0.5 ml of final formulations, a dose considered to be half a human dose. Guinea pigs received 5 ml, or five human doses. Animals were treated at an age where they were still growing and maintenance of their growth rate was the study endpoint. Groups size was two animals for all studies.
[f]This study was conducted in accordance with the Pyrogen Test Guidelines of the United States Pharmacopeia (USP) 23.
[g]Formulations of CRL1005 or CTP37-DT alone and mixed were evaluted in separate groups of rabbits, three rabbits/group.
[h]Doses were adjusted for administration on a weight basis (mg/kg).

formulations with 3-, 10- or 25-mg doses of CRL1005 copolymer (Fig. 12). The magnitude of these responses were comparable to or exceeded those induced in the previous study using the CFA-like adjuvant [40]. A surprise observation was that the lowest dose tested, 3 mg, was as effective as the 10- and 25-mg doses while the highest dose, 75 mg, was ineffective.

In vitro, cytokine production by peripheral blood lymphocytes following activation with vaccine antigens was measured to determine the effects of CRL1005 on cellular immune responses (Fig. 13). When intact βhCG was used to activate the lymphocytes, production and secretion of γ-IFN, IL-2, IL-5 and IL-10 were observed indicating a mixed Th1 and Th2 response. Cytokine production was treatment related since very low levels of cytokines were produced by lymphocytes taken from these same patients prior to vaccinations. As was seen for antibody responses, the 3-, 10- and 25-mg doses appeared to be optimal.

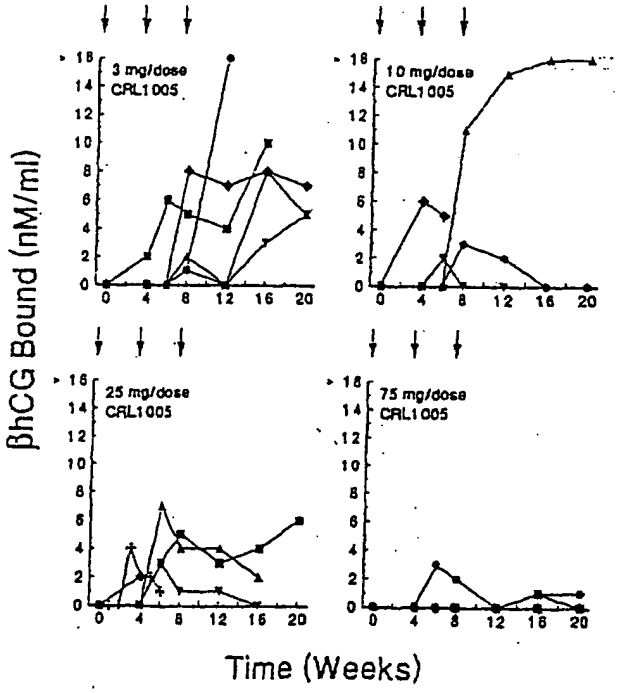

Fig. 12. The effect of CRL1005 copolymer in humans on antibody responses was evaluated in a Phase I clinical trial involving metastatic cancer patients. All formulations contained 1 mg/dose CTP37-DT and were supplemented with 3, 10, 25 or 75 mg/dose CRL1005. Immunizations were given at time point 0, 4 weeks and 8 weeks, final dose volume was 1 ml. Blood samples were collected at defined time points throughout the study. Levels of antibodies in serum specific for intact βhCG were measured by radioimmunoassay and the data are expressed as amount of βhCG bound (nM/ml). Antibody responses of patients who received up to three immunizations of CTP37-DT formulations supplemented with CRL1005 copolymer at 3, 10, 25 and 75 mg per dose are shown.

Similar cytokine measurements were done using DT but here all patients were known to have been vaccinated prior to the clinical trial (Fig. 14). Again, vaccine treatment increased the production of these cytokines, but patients in all dose groups responded similarly which meant that an effect of the CRL1005 adjuvant was not obvious. The patients who received 75 mg CRL1005 responded as well those who received the lower doses, which is different than was seen using βhCG to activate lymphocytes. Since these responses are memory recall responses, these data suggest that high doses of CRL1005 are not toxic to the immune system and the failure of high doses of CRL1005 to initiate responses may be a formulation problem.

The CRL1005 appears to be a very potent adjuvant in humans. The 3-mg dose is comparable to dose levels used in mice with OVA and the Fluogen® vaccines and it was included in the clinical testing because we thought it would be inactive and nontoxic. The fact that the highest dose was ineffective, leads us to believe an optimal ratio of CRL1005 and vaccine antigen is needed. The potency at lower doses and the lack toxicity at all dose levels support the continued development of this product for use in prophylactic vaccines.

5. Multiple emulsions for vaccine delivery

Multiple emulsions are three-phase, water-in-oil-in-water emulsions which are based on the use of both a high-MW copolymer with a low HLB, such as CRL1005, and a more hydrophilic copolymer, such as Pluronic® P123 [22]. Vaccine formulations are formed by the emulsification of antigen and the adjuvant-active, high-MW copolymer into a water-in-oil emulsion followed by the re-emulsification in aqueous media with the hydrophilic copolymer. In the final formulation, the oil content can be as low as 10% with the antigen entrapped in the inner-aqueous

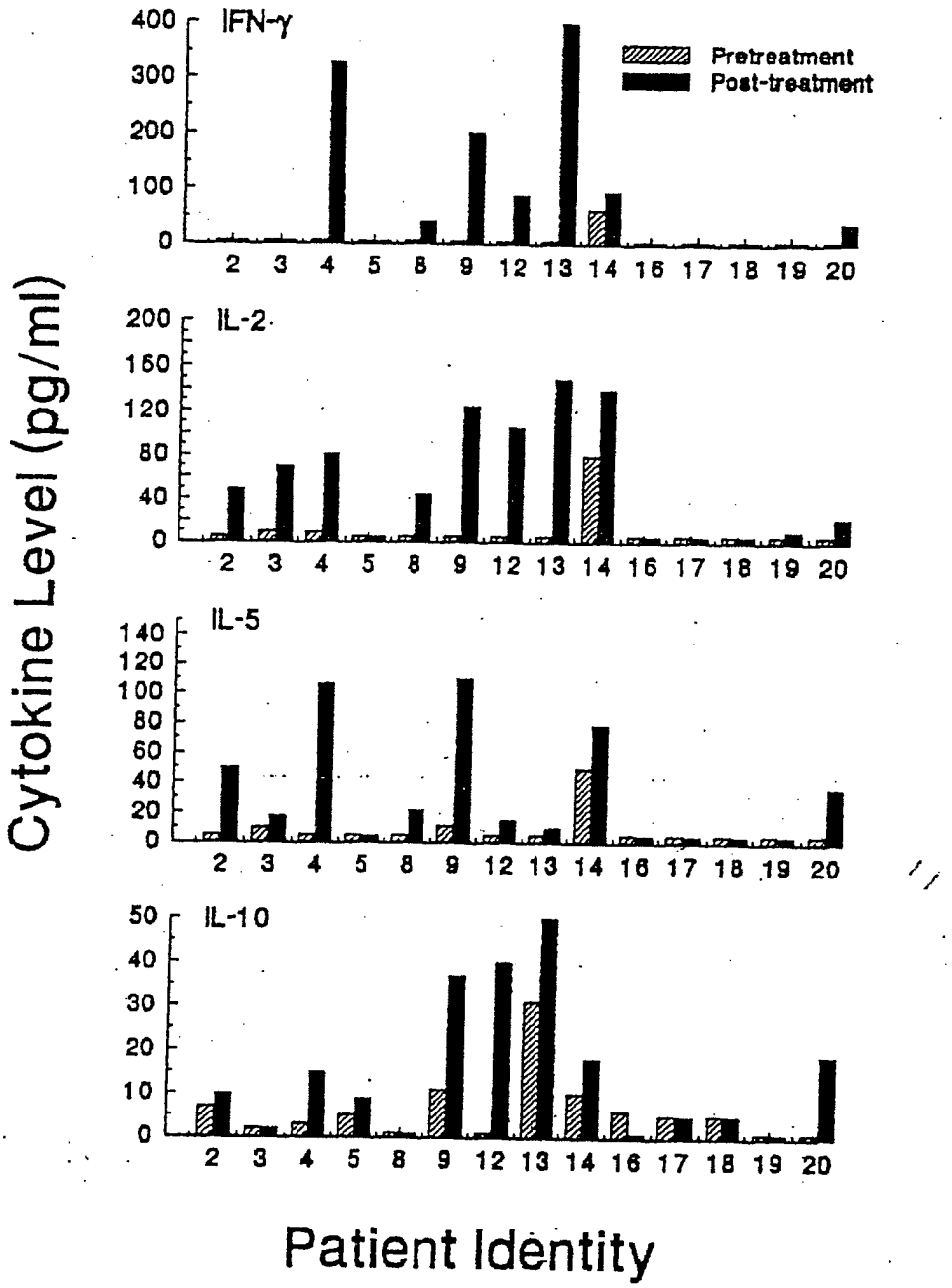

Fig. 13. Cytokine responses specific for were measured using peripheral blood lymphocytes obtained from patients prior to and after three immunizations with CTP37-DT-based vaccine formulations supplemented with 3, 10, 25 or 75 mg/dose CRL1005. Lymphocytes were cultured at the concentration of 2×10^6 with intact βhCG at the concentration of 25 μg/ml. Culture supernatant fluids were recovered and cytokine quantities (pg/ml) determined using a commercial antigen-capture ELISA and a standard curve. Sufficient peripheral blood lymphocytes were not available from all patients but representative patients from all CRL1005 dose groups were evaluated. Patients numbers 2–5 received formulations with 3 mg/dose CRL1005, patient numbers 6 and 8 received 10 mg/dose, patient numbers 12–14 received 25 mg/dose and 16–20 received 75 mg/dose.

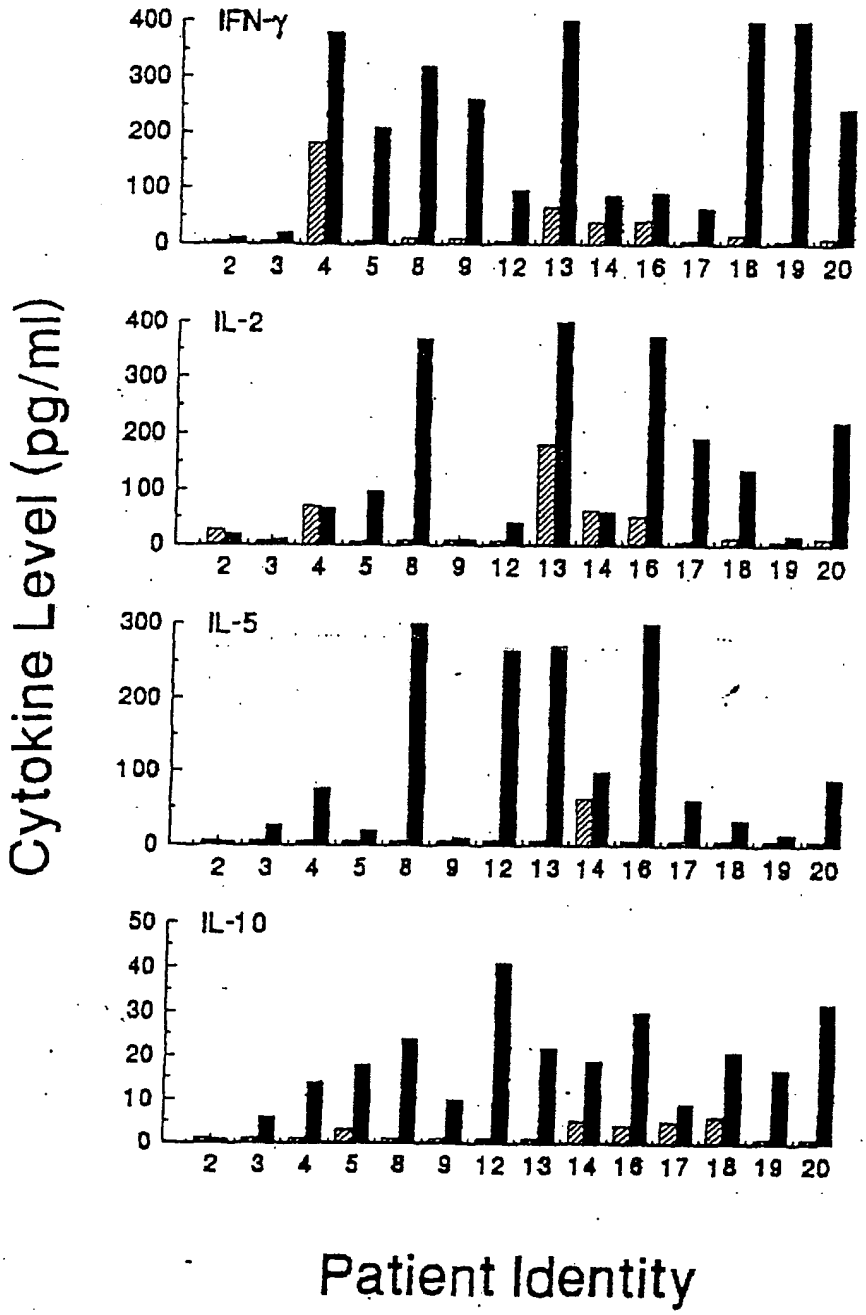

Fig. 14. Cytokine responses specific for DT were measured as reported using the same experimental conditions used to measure βhCG-specific cytokine production.

phase, the high MW copolymer aligned at the interface of the oil and inner aqueous interface and the hydrophilic copolymer aligned at the interface between the oil and outer-aqueous phases (Fig. 15).

High-MW copolymers in aqueous formulations have not proved useful for every vaccine antigen format. For example, soluble synthetic peptides do not always interact with the copolymers in a manner similar to large proteins and as such, the results from experiments using peptide-based antigens have been

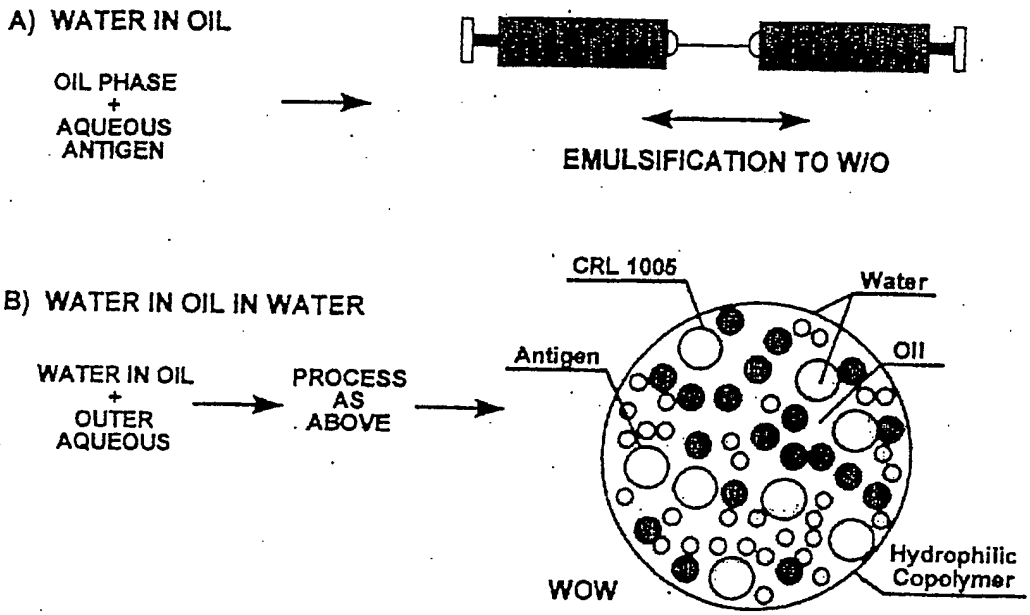

Fig. 15. Multiple emulsions are produced using a two-step emulsification process. Firstly, a water-in-oil emulsion is produced with the vaccine antigen in the aqueous phase and a hydrophobic copolymer, such as CRL1005, at the interface. This is then re-emulsified in a second aqueous phase with a hydrophilic copolymer to produce a water-in-oil-in-water emulsion.

variable. As was noted for the water-in-oil emulsion, TiterMax, peptides can be very immunogenic if delivered to the immune system properly. Multiple emulsions represent an alternative, copolymer-based vaccine delivery format that may be useful for addressing this limitation since a peptide vaccine can be presented to the immune system in the context of a water-in-oil formulation but without the high oil content and high viscosity associated with the standard water-in-oil emulsions.

Experimental parenteral vaccine formulations based on this format have been evaluated in laboratory animals and nonhuman primates and found to be very potent. For example, high titered antibody responses were induced in rhesus macaques using a multiple emulsion-based vaccine with inactivated SIV as the vaccine antigen [22,58]. The antibodies produced were capable of neutralizing SIV and protected some of the animals from infectious SIV challenge. These data suggest that vaccine antigens can be formulated into multiple emulsions and retain both their immunogenicity and appropriate conformation, similar to the oil-in-water emulsions described previously.

Vaccine antigens associated with copolymers in micelles should be compatible for oral or nasal delivery. Unfortunately, the copolymers provide little protection for protein antigens as they enter the stomach and there are no reasons to assume that copolymer micelles will be taken-up the lymphoid organs associated with mucosal tissues. Here is another potential use for multiple emulsions.

The experimental use of the multiple emulsion to deliver vaccines orally has provided some very interesting results. In studies using mice, mucosal and systemic antibody responses were induced by feeding vaccine antigen in multiple emulsions, provided cholera toxin was included in the inner aqueous phase [22,59]. The toxic effects of the cholera toxin was reduced significantly when delivered this way but the adjuvant effect was not lost. The mechanism behind this effect is unknown but we believe that two possibilities are likely. Firstly, the oil droplets may become widely dispersed throughout the intestine before the inner aqueous phase, containing the antigen and cholera toxin, is released. This would spread the cholera toxin over a much larger area than would be expected for normal oral gavage and this could reduce the localized toxicity. Secondly, the oil droplets may be taken-up by the gut epithelium so that the cholera toxin is released intracellularly. Cholera toxin, and related bacterial

toxins, are the most potent oral vaccine adjuvants yet identified and a delivery vehicle that would control their toxicity and thereby allow for their use in humans could be very beneficial.

The use of the multiple vehicle without cholera toxin appears more likely to induce oral immunological tolerance [60]. This oral tolerance is induced and maintained as an antigen-specific immune response where the final effect is the suppression of systemic immunity [61]. This type of tolerance has been demonstrated in several species, including humans [62,63], and has even been evaluated as an experimental therapy for human autoimmune disorders [64–67]. The quantities of antigen that are fed are large and as such, the therapeutic value of this treatment may limited. However, the use of multiple emulsions may allow for smaller quantities of antigen to be used or may induce more potent and long-lived oral tolerance. This oral vaccine vehicle may also prove useful for allergy desensitization and any other type of treatment where it is desirable to down-regulate pre-existing immune responses.

6. Conclusion

Nonionic block copolymers can be designed and manufactured with different physicochemical properties, simply by varying the ratio of the hydrophobic POP and hydrophilic POE components. Thus, it is possible to affect their compatibility with vaccine antigens and potency as vaccine adjuvants. The new high-MW copolymers, which can be manufactured and purified as uniform preparations, are nontoxic, can be used in oil-free formulations and are active in many species, including man. They can also be used in emulsion formulations as stabilizers or as carrier systems for other adjuvants, such as cholera toxin. These properties, coupled with the ability to selectively match individual copolymers with different vaccine antigens, make copolymers ideally suited for widespread use in the vaccine industry.

Acknowledgements

Fig. 5 is reprinted from *The Theory and Practical Application of Adjuvants*, Ref. [22], with the permission of John Wiley and Sons Ltd, Baffins Lane, Chishester, West Sussex, England. Figs. 7, 8, 10 and 11 were developed using data in *Mechanisms of Ageing and Development*, Ref. [47], with the permission of Elsevier Science Ireland Ltd., Bay 15K, Shannon Industrial Estate, Co. Clare, Ireland and *Vaccine*, Ref. [48] with the permission of Elsevier Science NL, Sara Burgerhartstraat 25, 1055 KV Amsterdam, The Netherlands.

References

[1] I.R. Schmolka, Polyalkylene oxide block copolymers, in: M.J. Schick (Ed.), Nonionic Surfactants, Marcel Dekker, New York, 1966, pp. 300–371.

[2] L.G. Lundsted, I.R. Schmolka, The synthesis and properties of block copolymer surfactants, in: R.J. Ceresa (Ed.), Block and Graft Copolymerization, John Wiley and Sons, London, 1976, pp. 1–111.

[3] I.R. Schmolka, Poloxamers in the pharmaceutical industry, in: P.J. Tarcha (Ed.), Polymers for Controlled Drug Delivery, CRC Press, Boca Raton, FL, 1991, pp. 189–214.

[4] M.M. Rieger, Surfactants, in: H.A. Lieberman, M.M., Rieger, M.M., G.S. Banker (Eds.), Pharmaceutical Dosage Forms: Disperse Systems, Marcel Dekker, New York, 1988, pp. 349–365.

[5] L.E. St. Pierre, Polymers from 1,2 epoxides: I. Chemistry, in: N.G. Gaylord (Ed.), Polyethers, Interscience, New York, 1963, pp. 83–168.

[6] A.S. Kastens, Polymers from 1,2 epoxides: II. Applications and technologies, in: N.G. Gaylord (Ed.), Polyethers, Interscience, New York, 1963, pp. 169–278.

[7] T.M. Schmitt, Analysis of Surfactants, Marcel Dekker, New York, 1992, pp. 1–464.

[8] L.G. Lundsted, I.R. Schmolka, The applications of block copolymer polyol surfactants, in: R.J. Ceresa (Ed.), Block and Graft Copolymerization, John Wiley and Sons, London, 1976, pp. 113–272.

[9] R.H. Whitmarsh, Synthesis and chemical modification of polyoxylalkylene block copolymers, in: V.M. Nace (Ed.), Nonionic Surfactants, Marcel Dekker, New York, 1996, pp. 1–30.

[10] D.A. Howerton, R.L. Hunter, H.K. Ziegler, I.J. Check, The induction of macrophage Ia expression in vivo by synthetic copolymer L81, J. Immunol. 144 (1990) 1578–1584.

[11] S.M. Moghimi, H. Hedeman, N.M. Christy, L. Illum, S.S. Davis, Enhanced hepatic clearance of intravenously administered sterically stabilized microspheres in zymosan-stimulated rats, J. Leukocyte Biol. 54 (1993) 513–517.

[12] S.M. Moghimi, J.C. Murray, Poloxamer-188 revisited: A potentially valuable immune modulator?, J. Natl. Cancer Inst. 88 (1996) 766–768.

[13] R.L. Hunter, B. Bennett, The adjuvant activity of nonionic block polymer surfactants. II. Antibody formation and inflammation related to the structure of triblocks and octablock copolymers, J. Immunol. 133 (1984) 3167–3175.

[14] P.D. Cooper, Solid phase activators of the alternative pathway of complement, in: R.M. Sim (Ed.), Activators and Inhibitors of Complement, Kluwer Academic Publishers, Dordrecht, 1993, pp. 69–106.

[15] P.D. Cooper, Vaccine adjuvants based on gamma inulin, in: M.F. Powell, M.J. Newman (Eds.), Vaccine Design. The Subunit and Adjuvant Approach, Plenum Press, New York, 1995, pp. 559–580.

[16] A.C. Allison, N.E. Byars, Syntex adjuvant formulation, Res. Immunol. 143 (1992) 519–525.

[17] A.C. Allison, N.E. Byars, Immunological adjuvants, Adv. Exp. Med. Biol. 327 (1992) 133–141.

[18] B. Waksman, Adjuvants and immune regulation by lymphoid cells, Springer Semin. Immunopathol. 23 (1979) 5–33.

[19] P. Jolles, A. Paraf, Chemical and biological basis of adjuvants, in: A. Kleinzeller, G. Springer, H. Wittmann (Eds.), Molecular Biology and Biophysics, Springer-Verlag, New York, 1973, pp. 1–153.

[20] D. Gall, Observations of the properties of adjuvants, in: R. Regamey, W. Hennessen, J. Ungar (Eds.), Symposia Series in Immunological Standardization, Karger, New York, 1967, pp. 39–48.

[21] R.L. Hunter, F. Strickland, F. Kezdy, Studies on the adjuvant activity of nonionic block polymer surfactants. I. The role of hydrophile-lipophile balance, J. Immunol. 127 (1981) 1244–1250.

[22] R.L. Hunter, M.R. Olsen, B. Bennett, Copolymer adjuvants and Titermax, in: D.E.S. Stewart-Tull (Ed.), The Theory and Practical Application of Adjuvants, John Wiley and Sons, New York, 1995, pp. 51–94.

[23] F.R. Vogel, M.F. Powell, A compendium of vaccine adjuvants and excipients, in: M.F. Powell, M.J Newman (Eds.), Vaccine Design: The Subunit and Adjuvant Approach, Plenum Press, New York, 1995, pp. 141–228.

[24] B. Bennett, I.J. Check, M.R. Olsen, R.L. Hunter, A comparison of commercially available adjuvants for use in research, J. Immunol. Methods 153 (1992) 31–40.

[25] W.M. Kast, R.M.P. Brandt, C.J.M. Melief, Strict peptide length is not required for the induction of cytotoxic T lymphocyte-mediated antiviral protection by peptide vaccination, Eur. J. Immunol. 23 (1993) 1189–1192.

[26] Y. Ke, Y. Li, J.A. Kapp, Ovalbumin injected with complete Freund's adjuvant stimulates cytolytic responses, Eur. J. Immunol. 25 (1995) 549–553.

[27] F. Rousset, S. Peyrol, E. Garcia, N. Vezzio, M. Andujar, J.-A. Grimaud, J. Banchereau, Long-term cultured CD40-activated B lymphocytes differentiate into plasma cells in response to IL-10 but not IL-4, Int. Immunol. 7 (1995) 1243–1253.

[28] D.M. Lidgate, N.E. Byars, Development of an emulsion-based muramyl dipeptide adjuvant formulation for vaccines, in: M.F. Powell, M.J. Newman (Eds.), Vaccine Design: The Subunit and Adjuvant Approach, Plenum Press, New York, 1995, pp. 313–324.

[29] S. Raychaudhuri, M. Tonks, F. Carbone, T. Ryskamp, W.J.W. Morrow, N. Hanna, Induction of antigen-specific class I-restricted cytotoxic T cells by soluble proteins in vivo, Proc. Natl. Acad. Sci. USA 89 (1992) 8308–8312.

[30] A.C. Allison, N.E. Byars, An adjuvant formulation that selectively elicits the formation of antibodies of protective isotypes and of cell mediated immunity, J. Immunol. Methods 95 (1986) 157–168.

[31] N.E. Byars, E.B. Fraser-Smith, R.A. Pecyk, M. Welch, G. Nakano, R.L. Burke, A.R. Hayward, A.C. Allison, Vaccinating guinea pigs with recombinant glycoprotein D of herpes simplex virus in an efficacious adjuvant formulation elicits protection against vaginal infection, Vaccine 12 (1994) 200–209.

[32] C.J. Issel, D.W. Horohov, D.F. Lea, W.V. Adams Jr., S.D. Hagius, J.M. McManus, A.C. Allison, R.C. Montelaro, Efficacy of inactivated whole-virus and subunit vaccines in preventing infection and disease caused by equine infectious anemia virus, J. Virol. 66 (1992) 3398–3408.

[33] R.C. Montelaro, C. Grund, M. Raabe, B. Woodson, R.F. Cook, S. Cook, C.J. Issel, Characterization of protective and enhancing immune responses to equine infectious anemia virus resulting from experimental vaccines, AIDS Res. Hum. Retroviruses 12 (1996) 413–415.

[34] M. Murphey-Corb, L.N. Martin, B. Davison-Fairburn, R.C. Montelaro, M.A. Miller, M. West, S. Ohkawa, G.B. Baskin, J.-Y. Zhang, A.C. Allison, D.A. Eppstein, A formalin-inactivated whoel SIV vaccine confers protection in macaques, Science 246 (1989) 1293–1297.

[35] R.G. Fenton, D.D. Taub, L.W. Kwak, M.R. Smith, D.L. Longo, Cytotoxic T-cell response and in vivo protection against tumor cells harboring activated ras proto-oncogenes, J. Natl. Cancer Inst. 85 (1993) 1294–1302.

[36] R.L. Hunter, B. Bennett, The adjuvant activity of nonionic block polymer surfactants. III. Characterization of selected biologically active surfaces, Scand. J. Immunol. 23 (1986) 287–300.

[37] E. Claassen, W. De Leeuw, P. De Greeve, C. Hendriksen, W. Boersma, Freund's complete adjuvant: An effective but disagreeable formula, Res. Immunol. 143 (1992) 478–483.

[38] M.R. Hilleman, Critical appraisal of emulsified oil adjuvants applied to viral vaccines, Prog. Med. Virol. 8 (1966) 131–151.

[39] M.R. Hilleman, A. Woohour, A. Friedman, R.E. Weibel, J. Stokes, The clinical application of adjuvant 65, Ann. Allergy 30 (1972) 152–158.

[40] P.L. Triozzi, D. Gochnour, E.W. Martin, W. Aldrich, J. Powell, J.A. Kim, D.C. Young, J. Lombardi, Clinical and immunologic effects of a synthetic β-human chorionic gonadotropin vaccine, Int. J. Cancer 5 (1994) 1447–1453.

[41] W. Keitel, R. Couch, N. Bond, S. Adair, G. Van Nest, C. Dekker, Pilot evaluation of influenza virus vaccine (IVV) combined with adjuvant, Vaccine 11 (1993) 909–913.

[42] C.J.M. Melief, R. Offringa, R.E.M. Toes, W.M. Kast, Peptide-based cancer vaccines, Curr. Opin. Immunol. 8 (1996) 651–657.

[43] B. Van den Eynde, V.G. Brichard, New tumor antigens recognized by T cells, Curr. Opin. Immunol. 7 (1995) 674–681.

[44] E. Celis, J. Fikes, P. Wentworth, J. Sidney, S. Southwood, A. Maewal, M.-F. Del Guercio, A. Sette, B. Livingston, Identifi-

cation of potential CTL epitopes of tumor-associated antigen MAGE-1 for five common HLA-A alleles, Mol. Immunol. 31 (1994) 1423–1430.

[45] A.P. Kendal, W.R. Dowdle, Influenza Virus, in: N.R. Rose, H. Friedman, J.L. Fahey (Eds.), Manual of Clinical Laboratory Immunology, American Society for Microbiology, Washington, DC, 1986, pp. 515–520.

[46] C.R. Kensil, J.Y. Wu, S. Soltysik, Structural and immunological characterization of the vaccine adjuvant QS-21, in: M.F. Powell, M.J. Newman (Eds.), Vaccine Design: The Subunit and Adjuvant Approach, Plenum Press, New York, 1996, pp. 525–542.

[47] M.J. Newman, C.W. Todd, E.M. Lee, M. Balusubramanian, P.J. Didier, J.M. Katz, Increasing the immunogenicity of a trivalent influenza virus vaccine with adjuvant-active nonionic block copolymers for potential use in the elderly, Mech. Ageing Dev. 93 (1997) 189–203.

[48] C.W. Todd, L.A.M. Pozzi, J.R. Guarnaccia, M. Balasubramanian, W.G. Henk, L.E. Younger, M.J. Newman, Development of an adjuvant active nonionic block copolymer for use in oil-free subunit vaccines formulations, Vaccine 15 (1997) 564–570.

[49] J.-Y. Wu, B.H. Gardner, C.I. Murphy, J.R. Seals, C.R. Kensil, J. Recchia, G.A. Beltz, G.W. Newman, M.J. Newman, Saponin adjuvant enhancement of antigen-specific immune responses to an experimental HIV-1 vaccine, J. Immunol. 148 (1992) 1519–1525.

[50] M.J. Newman, J.-Y. Wu, B.H. Gardner, K.J. Munroe, D. Leombruno, J. Recchia, C.R. Kensil, R.T. Coughlin, Saponin adjuvant induction of ovalbumin-specific $CD8^+$ cytotoxic T lymphocyte responses, J. Immunol. 148 (1992) 2357–2362.

[51] H. Takahashi, T. Takeshita, B. Morein, S. Putney, R.N. Germain, J.A. Berzofsky, Induction of CD8 + cytotoxic T cells by immunization with purified HIV-1 envelope protein in ISCOMs, Nature 344 (1990) 873–875.

[52] B. Rönnberg, M. Fekadu, B. Morein, Adjuvant activity of non-toxic *Quillaja suponaria* Molina components for use in ISCOM matrix, Vaccine 13 (1995) 1375–1382.

[53] L. Tarpey, S.N. Stacey, A. McIndoe, D.H. Davies, Priming in vivo and quantification in vitro of class I MHC-restricted cytotoxic T cells to human papilloma virus type 11 early proteins (E6 and E7) using immunostimulating complexes (ISCOMs), Vaccine 14 (1996) 230–236.

[54] C.V. Harding, D. Collins, E.R. Unanue, Processing of liposome-encapsulated antigens targeted to specific subcellular compartments, Res. Immunol. 143 (1992) 188–191.

[55] Y. Yasutomi, S. Koenig, R.M. Woods, J. Madsen, N.M. Wassef, C.R. Alving, H.J. Klein, T.E. Nolan, L.J. Boots, J.A. Kessler, E.A. Emini, A.J. Conley, N.L. Letvin, A vaccine-elicited, single viral epitope-specific cytotoxic T lymphocyte response does not protect against intravenous, cell-free simian immunodeficiency virus challenge, J. Virol. 69 (1995) 2279–2284.

[56] D.S. Collins, K. Findlay, C.V. Harding, Processing of exogenous liposome-encapsulated antigens in vivo generates class I MHC-restricted T cell responses, J. Immunol. 148 (1992) 3336–3341.

[57] P.L. Triozzi, V.C. Stevens, W. Aldrich, J. Powell, C.W. Todd, M.J. Newman, Effects of a β-human chorionic gonadotropin subunit immunogen administered in aqueous solution with a novel nonionic block copolymer adjuvant in patients with advanced cancer, Clin. Cancer Res. 3 (1997) 2355–2362.

[58] P. Millet, M. Kalish, W.E. Collins, R.L. Hunter, Effect of adjuvant formulations on the selection of B-cell epitopes expressed by a malarial peptide vaccine, Vaccine 10 (1992) 547–550.

[59] M. Tomasi, M.T. Dertzbaugh, T.L. Hearn, R.L. Hunter, C.O. Elson, Strong mucosal adjuvanticity of cholera toxin within lipid particles of a new multiple emulsion delivery system for oral immunization, Eur. J. Immunol. 27 (1997) 2720–2725.

[60] C.O. Elson, M. Tomasi, M. Dertzbaugh, G. Thaggard, R.L. Hunter, C. Weaver, Oral-antigen delivery by way of a multiple emulsion system enhances oral tolerance, Ann. NY Acad. Sci. 778 (1996) 156–162.

[61] A. Miller, O. Lider, A.B. Roberts, M. Sporn, H.L. Weiner, Suppressor T cells generated by oral tolerization to myelin basic protein suppresses both in vitro and in vivo immune responses by the release of TGF-b following antigen-specific triggering, Proc. Natl. Acad. Sci. USA 89 (1992) 421–425.

[62] S. Husby, J. Mestecky, Z. Moldoveanu, S. Holland, C.O. Elson, Oral tolerance in humans. T cell but not B cell tolerance after antigen feeding, J. Immunol. 152 (1994) 4663–4670.

[63] Y. Chen, J. Inobe, H.L. Weiner, Induction of oral tolerance to myelin basic protein in CD8-depleted mice: Both $CD4^+$ and $CD8^+$ cells mediate active suppression, J. Immunol. 155 (1995) 910–916.

[64] H.L. Weiner, G.A. Mackin, M. Matsui, E.J. Orav, S.J. Khoury, D.M. Dawson, D.A. Hafler, Double-blind pilot trial of oral tolerization with myelin antigens in multiple sclerosis, Science 259 (1993) 1321–1324.

[65] D.E. Trentham, R.A. Dynesius-Trentham, E.J. Orav, D. Combitchi, C. Lorenzo, K.L. Sewell, D.A. Hafler, H.L. Weiner, Effects of oral administration of type II collagen on rheumatoid arthritis, Science 261 (1993) 1727–1730.

[66] H.L. Weiner, A. Miller, S.J. Khoury, Z.J. Zhang, A. Al-Sabbagh, S.A. Brod, O. Lider, P. Higgins, R. Sobel, M. Matsui, M. Sayegh, C. Carpenter, G. Eisenbarth, R.B. Nussenblatt, D.A. Hafler, Treatment of autoimmune diseases by oral tolerance to autoantigens, Adv. Exp. Med. Biol. 371B(PT 2) (1995) 1217–1223.

[67] H.L. Weiner, A. Friedman, A. Miller, S.J. Khoury, A. Al-Sabbagh, L. Santos, M. Sayegh, R.B. Nussenblatt, D.E. Trentham, D.A. Hafler, Oral tolerance: Immunolgoical mechanisms and treatment of animal and human organ-specific autoimmune diseases by oral administration of autoantigens, Ann. Rev. Immunol. 12 (1994) 809–837.

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re the Application of:	Group Art Unit 1648
BLONDER et al.	Examiner: Li, Bao Q.
Serial No.: 09/888,235	RULE 132 DECLARATION OF CLAIRE M. COESHOTT (37 C.F.R. § 1.132)
Filed: June 22, 2001	
Atty. File No.: 42830-00234	
For: "DELIVERY VEHICLE COMPOSITION AND METHODS FOR DELIVERING ANTIGENS AND OTHER DRUGS"	

CERTIFICATE OF MAILING

I HEREBY CERTIFY THAT THIS CORRESPONDENCE IS BEING DEPOSITED WITH THE UNITED STATES POSTAL SERVICE AS FIRST CLASS MAIL IN AN ENVELOPE ADDRESSED TO ASSISTANT COMMISSIONER FOR PATENTS, WASHINGTON, DC 20231 ON April 22, 2003.

MARSH FISCHMANN & BREYFOGLE, LLP

BY: [signature]

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Madam:

Claire M. Coeshott, residing at 875 South Josephine Street, Denver, Colorado 80209 80027, declare as follows:

I am currently employed in the capacity of Director, Vaccine Technologies by RxKinetix, Inc., the assignee of the referenced U.S. Patent Application.

The attached Exhibit A is a summary of my technical qualifications.

The attached Exhibit B summarizes some tests (identified as Examples 11-16 for convenient reference) performed by me or by others at my direction concerning compositions for delivery of antigens. Examples 11-16 presented in Exhibit B concern formulation and testing of antigen delivery test compositions in which the antigen is formulated in an aqueous liquid with an adjuvant material and a polymer of a type and in an amount to impart reverse-thermal viscosity behavior to the composition. Antigens subject to the testing include tetanus toxoid (TT), diphtheria toxoid (DT) and recombinant anthrax protective antigen (rPA); adjuvant materials tested include those containing chitosan or CpG dinucleotide motifs (CpG); and the polymer for all tests is Pluronic® F127 polymer. Studies in mice compared the performance of these test compositions as compared to comparison compositions in which the antigen is differently formulated. Results of these mice studies are

discussed in Exhibit B, with tabular results of mice antibody response data for Examples 12-15 being presented in attached Exhibits C-F. The results of the mice studies presented in Exhibits B-F demonstrate a high level of antibody response to the test composition, and with the antibody response to the test composition most often being both faster to develop and attaining a higher level than the antibody response to the comparison compositions, as indicated by antibody assays. The attainment of a higher level of antibody response is obviously important. Perhaps more important, however, is the faster antibody response to the test compositions. In a high-risk situation, such as an epidemic, development of quicker immunization response following antigen administration may mean the difference between someone surviving or not surviving the situation. This more rapid response to immunization is surprising as it might be expected that administering the antigen in the reverse-thermal viscosity composition would delay distribution of the antigen to the relevant cells of the immune system, thus slowing any immune response.

Example 11 presents a general procedure for preparing formulations and for performing and obtaining antibody assays to determine antibody response. Examples 12-16 discuss preparation of specific formulations and mice studies on those particular formulations, generally as described in Example 11 except as noted.

In Example 12, test compositions with TT are formulated with 16.25% (w/w) Pluronic®F127 polymer and with varying amounts of an adjuvant material containing chitosan (0.5, 0.17, or 0.05% (w/w) of the adjuvant material). Comparative compositions with TT are also formulated with only the adjuvant material or with only the polymer. The test compositions demonstrate higher IgG antibody response at both two weeks and at five weeks following a single subcutaneous administration of 0.5 Lf TT than the comparable comparative compositions, as clearly summarized in the following table, which provides data for geometric mean and average IgG antibody titers in serum samples from the mice studies on the different compositions.

Chitosan Adjuvant Material Content	IgG Antibody Titers – Geometric Mean and (Average)		
	Test Comp. With Both Adj. Mtl. & Polymer	Comparative Comp. With Only Adj. Mtl.	Comparative Comp. With Only Polymer
Two Weeks Following Administration			
0.5% (w/w)	413 (445)	162 (396)	
0.17% (w/w)	497 (665)	337 (467)	
0.05% (w/w)	252 (271)	215 (236)	
0% (w/w)			27 (56)
Five Weeks Following Administration			
0.5% (w/w)	14,132 (18,836)	4748 (5403)	
0.17% (w/w)	11,201 (13,194)	9,119 (11,442)	
0.05% (w/w)	5,437 (7,055)	4,862 (6,165	
0% (w/w)			122 (289)

As summarized in the above table, the comparative composition formulated with only Pluronic® F127 polymer, and no adjuvant material, performed poorly. Comparative compositions formulated with only the adjuvant material, and no Pluronic® F127 polymer performed better than comparative compositions formulated with only Pluronic® F127 polymer, but the test compositions, formulated with both the adjuvant material and the Pluronic® F127 polymer, performed the best.

In Example 13, test compositions with TT are formulated with 16.25% (w/w) Pluronic® F127 polymer and with 20% (v/w) adjuvant material containing CpG. Comparative compositions with TT are also formulated without the Pluronic® F127 polymer, but with the CpG-containing adjuvant material with and without the addition also of glycerol or incomplete Freund's adjuvant (IFA). The test compositions demonstrate higher IgG antibody response following a single subcutaneous administration of 0.5 Lf TT than the comparative compositions. It is of particular interest to point out that IFA is considered a "gold standard" for adjuvants used in immunization of experimental animals and that the test composition is an improvement. In Example 14, test compositions with TT are formulated with 16.25% (w/w) Pluronic® F127 polymer and with various amounts of an adjuvant material containing CpG (20, 6.7 or 2 % (v/w) of the adjuvant material). Comparative

compositions with TT are also formulated with only the adjuvant material or with only the polymer. The test compositions consistently demonstrate higher IgG antibody response at two, four and eight weeks following a single subcutaneous administration of 0.5 Lf TT than the comparable comparative compositions, as clearly summarized in the following table, which provides data for geometric mean and average IgG antibody titers in serum samples from the mice studies on the different compositions.

CpG Adjuvant Material Content	IgG Antibody Titers – Geometric Mean and (Average)		
	Test Comp. With Both Adj. Mtl. & Polymer	Comparative Comp. With Only Adj. Mtl.	Comparative Comp. With Only Polymer
Two Weeks Following Administration			
20%(v/w)	6,974 (7,705)	5,287 (5,792)	
6.7% (v/w)	1,761 (1,969)	476 (554)	
2% (v/w)	694 (792)	264 (284)	
0% (vw)			623 (780)
Four Weeks Following Administration			
20%(v/w)	14,768 (32,636)	6,050 (8,309)	
6.7% (v/w)	77,632 (101,667)	3,225 (3,472)	
2% (v/w)	14,037 (18,054)	2,243 (2,282)	
0% (v/w)			626 (884)
Eight Weeks Following Administration			
20%(v/w)	39,903(77,778)	14,429(46,467)	
6.7% (v/w)	76,792(172,083)	8,566(11,619)	
2% (v/w)	17,065(27,739)	4,034(4,714)	
0% (v/w)			345(926)

As summarized in the above table, the comparative composition formulated with only Pluronic® F127 polymer, and no adjuvant material, performed poorly. Comparative compositions formulated with only the adjuvant material, and no Pluronic® F127 polymer performed better than comparative compositions formulated with only Pluronic® F127 polymer, but the test compositions, formulated with both the adjuvant material and the Pluronic®F127 polymer, consistently performed the best. The

values for the 20% test composition may be lower than expected in this example due to technical difficulties performing the assay.

In Example 15, test compositions with DT are formulated with 16.25% (w/w) Pluronic®F127 polymer and with 20% (v/w) adjuvant material containing CpG. Comparative compositions with DT are also formulated without the Pluronic® F127 polymer, but with the adjuvant material containing CpG. The test compositions demonstrate attainment of a higher IgG antibody response following a single subcutaneous administration of 1 Lf DT than the comparative compositions, although at later times (after 12 weeks) following administration, the comparative compositions do result in similiar IgG antibody responses.

In Example 16, test compositions with rPA are formulated with 16.25% (w/w) Pluronic® F127 polymer and with 20% (v/w) adjuvant material containing CpG. Comparative compositions with rPA are also formulated without the Pluronic® F127 polymer, but with either the CpG-containing adjuvant material or alternatively with aluminum hydroxide (alum). The test compositions demonstrate attainment of a higher IgG antibody response following a single subcutaneous administration of 25 μg rPA than the comparative compositions including the alum. Also, the test compositions resulted in significantly higher toxin neutralization antibody titers than either the comparison compositions with the CpG-containing adjuvant or the comparison compositions containing alum. The toxin neutralization assay is a measure of the ability of the test composition to raise an antibody response that protects cells against challenge with anthrax lethal toxin and therefore is an excellent indicator of the effectiveness of the test composition.

All statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true. I understand that willful false statements and the like are punishable by fine or imprisonment, or both (18 U.S.C. §1001) and may jeopardize the validity of this patent application or any patent issuing thereon.

Respectfully submitted,

Date: 4/22/03

By: C M Coeshott

Claire M. Coeshott

EXHIBIT A
TO RULE 132 DECLARATION OF
CLAIRE M. COESHOTT

BIOGRAPHICAL SKETCH AND TECHNICAL QUALIFICATIONS

NAME Coeshott, Claire M.	TITLE Director, Vaccine Technologies

EDUCATION/TRAINING

INSTITUTION AND LOCATION	DEGREE (*if applicable*)	YEAR(s)	FIELD OF STUDY
University of Bristol, England	B.Sc., 1st class, honors	1978	Pathology
University of Bristol, England	Ph.D.	1982	Immunology

RESEARCH AND PROFESSIONAL EXPERIENCE:

Employment

1981-1982 Research Assistant, Department of Pathology, University of Bristol, England.

1982-1985 Research Fellow, National Jewish Hospital and Research Center, Denver, Colorado.

1985-1988 Research Associate, Division of Membrane Biology, Medical Biology Institute, La Jolla, California.

1988-1994 Group Leader: Immunology, Cortech Inc., Denver, Colorado.

1991-1994 Team Leader: Lupus Project, Cortech Inc., Denver, Colorado.

1994-1996 Group Leader: Immunoassay Methods, Biopharmaceutics Department, Cortech Inc., Denver, Colorado.

1996-1997 Group Leader: Protease Inhibitor Program, Pharmacology Department, Cortech Inc., Denver, Colorado.

1997-1998 Research Fellow and Group Leader, Biology Department, Ribozyme Pharmaceuticals Inc., Boulder, Colorado.

1999-2000 Senior Scientist, Ceres Pharmaceuticals Ltd., Denver, Colorado.

2000-2002 Senior Scientist, RxKinetix Inc. Louisville, Colorado.

2002 Director, Vaccine Technologies, RxKinetix Inc. Louisville, Colorado.

Synopsis Of Industrial Experience

RxKinetix Inc. 2000 – present

Project leader for vaccine program to evaluate proprietary formulations for vaccine delivery. Coordinate research effort in house and with outside collaborators. Develop

assays for measurement of antibody and T cell responses to formulations. Liaise with business development and legal departments for optimal positioning of technology.

Globeimmune, Inc (formerly Ceres Pharmaceuticals Ltd.) 1999 – 2000
Employed as both bench scientist and manager for a SBIR-funded project to develop a genetically-engineered microorganism as an HIV vaccine. Designed and executed *in vivo* and *in vitro* experiments for vaccine program:

- obtained Proof of Principle for vaccine candidate using a tumor protection model in mice.

Ribozyme Pharmaceuticals Inc. 1997 – 1998
Led multidisciplinary project to develop ribozyme-based therapeutic to treat chemoresistance in cancer. Team consisted of 3 Ph.Ds. and 4 RAs. In addition, was line manager for 3 RAs within Biology Group:

- co-ordinated synthesis of ribozymes, designed in vitro experiments with RNA endpoints (RNase protection assay and Taqman analysis) and phenotypic endpoints (apoptosis).
- designed and oversaw in vivo experiments to test lead compounds using human cancer cell line xenografts in athymic mice

Cortech Inc. 1989 – 1997
Immunology Program: Basic Research
Developed Immunology program using multivalent arrays of haptens on large molecular weight carriers such as dextran to suppress or stimulate hapten-specific antibody responses in mice. Outcomes of program:

- patent issued (November 1996) addressing stimulatory aspects of technology which formed basis of vaccine program at Cortech.
- filing of an IND application (March 1995) and subsequent completion of phase I clinical trial for a specific immunomodulator, CI-0694, to suppress sulfamethoxazole hypersensitivity in AIDS patients .

Set up tissue culture laboratory as service facility for providing monoclonal antibodies to other projects:

- developed and characterized peptide-specific helper T cell hybridomas and their responses to various Cortech compounds.
- demonstrated activity of Cortech compounds for cytotoxic T cell induction.
- developed and characterized monoclonal antibodies against fibrinopeptides and bradykinin antagonists.

Protease Inhibitor Program: Research
Program addressed potential of novel synthetic, substrate-based compounds to inhibit enzymatic degradation of tissues and release of various cytokines:

- designed and executed assays for measuring impact of inhibitors on cytokine production (TNFα, IL-1β, IL-2, IL-8) from whole blood as well as from various cell types including THP-1 monocytic cell line, Jurkat, neutrophils and monocytes isolated from human peripheral blood.
- oversaw development of extracellular matrix assay to test inhibition of radiolabelled matrix degradation.

Managerial

As leader of Lupus project, coordinated a team of up to three Ph.Ds. and four RAs in the production of compound to suppress nephritis occurring in the autoimmune disease, systemic lupus erythematosus:

- initiated and oversaw collaborations with researchers in field to assess recognition of Cortech compounds by antibodies from human SLE patients.
- developed ELISPOT assay to measure anti-DNA and anti-histone antibody-secreting cells.
- designed and executed all in vivo experiments to monitor the effects of these constructs in lupus-prone mice.
- lead compound identified.

Pre-clinical Research

As member of Biopharmaceutics department, supervised two senior- level RAs and one post-doctoral researcher:

- developed immunoassays to measure specific antibody responses in AIDS patients entering phase I clinical trial of CI-0694. ELISA and competition ELISA for IgM, IgA and IgG developed and subsequently used for measurement of antibodies in samples from phase I trial. Liased with AIDS Clinical Trial Group (ACTG) in evaluation of CI-0694.
- collaborated with physicians at Denver General Hospital in study to investigate correlation between antibody levels and failure of desensitization to sulfamethoxazole.
- coordinated clinical studies to examine efficacy of elastase inhibitor, CE-1037, in cystic fibrosis and ARDS: defined sample handling procedures for BALF and sputum; participated in site visits and initiation of two clinical trials.
- wrote research reports and SOPs; reviewed INDs, clinical protocols and other documents.

Awards, Honors, Grants

1. Leukemia Society of America Special Fellowship, July 1987 - July 1990.
2. University of Bristol Postgraduate Scholarship, 1978 - 1981.

Memberships

British Society for Immunology

Patents

1 issued; 2 applications

Selected Publications

Grace S.A., Elson, C.J. and Coeshott, C.M. Production of anti-host IgG by transfer of primed histocompatible cells. **Clin. Exp. Immunol. 39:449, 1980.**

Elson, C.J. and Coeshott, C.M. Tolerance of allotypic determinants induced by lymphoid cells from congenic mice bearing the allotype. **Immunol. 43:281, 1981.**

Coeshott, C.M. and Grey, H.M. Transfer of antigen presenting capacity to la negative cells upon fusion with la-bearing liposomes. **J. Immunol. 134:1343, 1985.**
Gay, D., Coeshott, C.M., Golde, W., Kappler, J. and Marrack, P. The Major Histocompatibility Complex-restricted antigen receptor on T cells IX. Role of accessory molecules in recognition of antigen plus isolated IA. **J. Immunol. 136:2026, 1986.**
Coeshott, C.M., Chesnut, R.W., Kubo, R.T., Grammer, S.F., Jenis, D.M. and Grey, H.M. Ia-specific mixed leukocyte reactive T cell hybridomas: Analysis of their specificity by using purified class II MHC molecules in a synthetic membrane system. **J. Immunol. 136:2832, 1986.**
Blodgett, J.K., Coeshott, C.M., Roper, E.F., Ohnemus, C., Allen, L.G., Kotzin, B.L. and Cheronis, J.C. Synthesis and characterization of novel antigen-specific immunosuppressive agents and their utilization in the (NZB x NZW)F1 murine model of systemic lupus erythematosus. **Proc. Amer. Pep. Symp. 12: 873, 1992.**
Coeshott, C., Allen, L., McLeod, D., Cheronis, J. and Kotzin, B. Antigen-specific suppression of antibody responses: implications for vaccine design. **Vaccines 95. Cold Spring Harbor Laboratory Press, 1995.**
De la Cruz, V.F., Cook, C., Allen, L., Strong, P., Blodgett, J., Ohnemus, C., McCall, C., Goodfellow, V., McLeod, D., Gross, K., Cheronis, J. and Coeshott, C. Antigen-specific Immunomodulation (ASIM): the rational design of molecules that are inherently immunogenic. **Vaccines 95. Cold Spring Harbor Laboratory Press, 1995.**
Pilyavskaya, A., Wieczorek, M., Asztalos, J., Coeshott, C., Francis, M.D. and Blodgett, J. Purification of $F(ab')_2$ and Fab' fragments from the T cell receptor-specific monoclonal antibodies, F23.1 and KJ16, and preparation of conjugates with dexamine. **J. International Bio-chromatography, 3: 215, 1996.**
Coeshott, C., Ohnemus, C., Pilyavskaya, A., Ross, S.E., Wieczorek, M. Kroona, H., Leimer, A. and Cheronis, J. Converting enzyme-independent release of TNFα and IL-1β from stimulated THP-1, a human monocytic cell line, in the presence of activated neutrophils or purified proteinase 3. **Proc. Natl. Acad. Sci. USA, 96: 6261, 1999.**
Stubbs, A.C., Martin, K.S., Coeshott, C., Skaates, S.V., Kuritzkes, D.R., Bellgrau, D., Franzusoff, A., Duke, R.C. and Wilson, C.C. Whole recombinant yeast vaccine activates dendritic cells and elicits protective cell-mediated immunity. **Nature Medicine 7:625-629, 2001.**
Westerink, M.A.J., Smithson, S.L., Srivastava, N., Blonder, J., Coeshott, C., and Rosenthal, G.J. Projuvant™ (Pluronic F127®/chitosan) enhances the immune response to intranasally administered tetanus toxoid. **Vaccine 20: 711-723, 2001.**

EXHIBIT B
TO RULE 132 DECLARATION OF
CLAIRE M. COESHOTT

EXAMPLE 11: General procedure for preparing and testing antigen delivery compositions

Preparation of formulations: Pluronic® F127 polymer (National Formulary pharmaceutical grade, BASF, Washington, NJ) stock solution was prepared at 34% (w/w) by dissolving in ice-cold PBS with complete dissolution achieved by storing overnight (ON) at 4°C. Protasan® (Chitosan chloride, ultrapure CL 213; Pronova Biomedical, Oslo, Norway; MW = 272,000; 84% deacetylated) stock solutions were prepared at 3% (w/w) in 1.0 % (v/v) acetic acid in sterile water (USP grade) and were heated at 37°C to dissolve. An adjuvant containing CpG dinucleotide motifs (CpG) was obtained from Qiagen (ImmunEasy™, proprietary formulation containing CpG of Qiagen Inc. Valencia, CA) and was added to formulations according to the manufacturer's instructions. The antigens evaluated include recombinant anthrax protective antigen (rPA), tetanus toxoid (TT), and diphtheria toxoid (DT). Adjuvants, such as those containing chitosan or CpG, were also added to the formulations. Unless otherwise noted, the stock solutions were mixed together to prepare formulations containing various combinations of antigen, adjuvant and Pluronic® F127 polymer.

Immunization studies in mice: Balb/c female mice (Harlan, Indianapolis, IN) 6 to 8 weeks of age were used for these studies. Groups of mice were immunized once subcutaneously (s.c.) with antigens in various formulations on day 0.

Antibody assays: The serum antibody responses to antigens were measured by ELISA. Wells of 96 well Nunc Maxisorb microtiter plates (Nunc, Gaithersburg, MD) were coated with the appropriate concentration of antigen in PBS. Plates were washed with PBS/0.05% Tween 20 and blocked with 1% bovine serum albumin (BSA) (Fisher Scientific, Pittsburgh, PA). Serum samples were serially diluted in PBS/0.1% BSA/0.05% Tween 20 (PBST) and added to wells in triplicate. Following incubation, plates were washed and goat anti-mouse IgG γ chain specific horseradish peroxidase (HRP)-labeled conjugate (Southern Biotechnology Associates Inc., Birmingham, AL) was added in PBST. After further incubation, antibody binding was detected with substrate buffer containing tetramethylbenzidine (TMB) (Sigma-Aldrich). Absorbance was read with an EIA reader (Molecular Devices, Sunnyvale, CA). Antibody titer was defined as the reciprocal of the dilution of serum that would yield an optical density of 0.5.

Statistics: Data were analyzed for differences using Students t test. A probability (p) of 0.05 or less was considered significant. Outliers were identified by Grubb's test.

Testing of performance of specific formulations with antigens TT, DT and rPA are discussed below in Examples 12-16.

EXAMPLE 12: TT with chitosan-containing adjuvant in the composition

Preparation of formulations: TT and Pluronic® F127 stock solutions were prepared as described in Example 1. Protasan® stock solution was prepared at 3% (w/w) in 1.0 % (v/v) acetic acid in sterile water (USP grade). Tetanus toxoid (Accurate Chemical & Scientific, Westbury, NY)

contained 1058 Lf/ml and 2204 Lf/mg protein nitrogen. The various stock solutions were mixed together to form vaccine compositions for testing, as follows:

(i) TT (5 Lf/ml), 0.5% (w/w) chitosan and 16.25% (w/w) Pluronic® F127;
(ii) TT (5 Lf/ml), 0.17% (w/w) chitosan and 16.25% (w/w) Pluronic® F127;
(iii) TT (5 Lf/ml), 0.05% (w/w) chitosan and 16.25% (w/w) Pluronic® F127;
(iv) TT (5 Lf/ml) and 0.5% (w/w) chitosan (no Pluronic® F127);
(v) TT (5 Lf/ml) and 0.17% (w/w) chitosan (no Pluronic® F127);
(vi) TT (5 Lf/ml) and 0.05% (w/w) chitosan (no Pluronic® F127); and
(vii) TT (5 Lf/ml) and 16.25% (w/w) Pluronic® F127 (no chitosan).

Immunization studies in mice: Balb/c female mice (Harlan), 6 to 8 weeks of age, were used for these studies. Mice were immunized once s.c with 0.5 Lf TT in the various formulations on day 0.

Antibody assays: The serum antibody responses to TT were measured by ELISA. Wells of 96 well Nunc Maxisorb microtiter plates (Nunc, Gaithersburg, MD) were coated with 1 μg/ml TT in PBS. Plates were washed with PBS/0.05% Tween 20 and blocked with 1% bovine serum albumin (BSA) (Fisher Scientific, Pittsburgh, PA). Samples were serially diluted in PBS/0.1% BSA/0.05% Tween 20 (PBST) and added to wells in triplicate. Following incubation, plates were washed and goat anti-mouse IgG γ chain specific horseradish peroxidase (HRP) labeled conjugate (Southern Biotechnology Associates Inc., Birmingham, AL) was added in PBST. After further incubation, antibody binding was detected with substrate buffer containing TMB (Sigma-Aldrich). Absorbance was read with an EIA reader (Molecular Devices, Sunnyvale, CA). Antibody titer was defined as the reciprocal of the dilution of serum that would yield an optical density of 0.5.

Serum samples were collected at weeks 2 and 5, and analyzed for IgG anti-TT antibodies by ELISA. The numerical IgG antibody titer data taken two weeks and five weeks following administration is presented in Exhibit C. At five weeks after a single injection, the response in animals receiving TT/F127/chitosan was significantly higher than that to TT in either component alone ($p = 0.02$ vs. TT/chitosan and $p = 0.0006$ vs. TT/F127) with the outlier removed. Figure 13 graphically summarizes IgG antibody titer data for tests on compositions (i), (iv) and (vii).

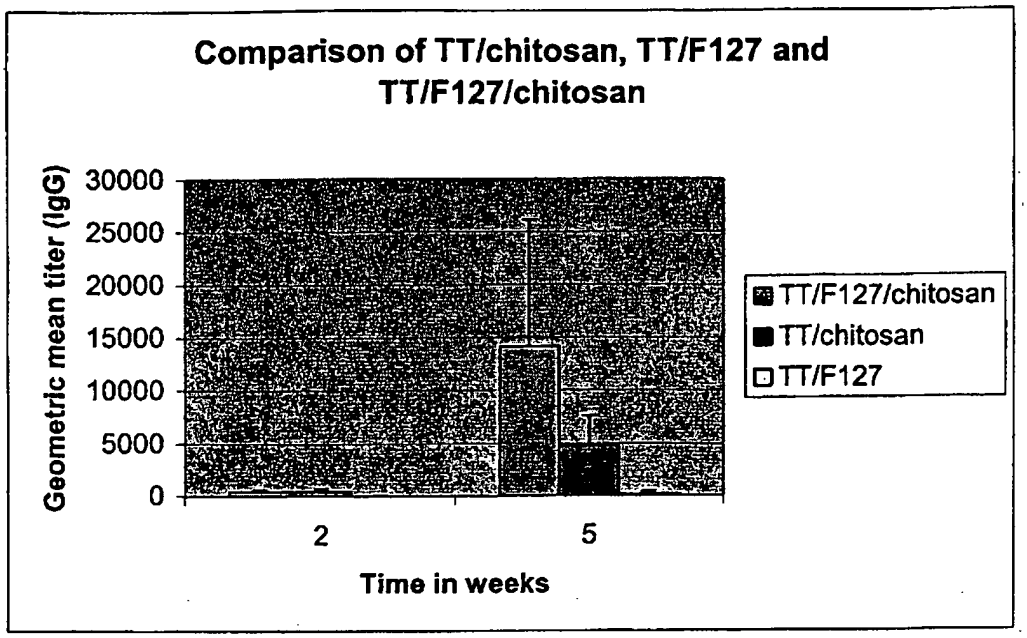

FIGURE 13

EXAMPLE 13: TT with CpG-containing adjuvant in the composition and comparison to other delivery vehicles

Preparation of formulations:. TT and Pluronic® F127 stock solutions were prepared as described in Example 1. ImmunEasy™ containing CpG as an adjuvant was added to formulations in an amount to provide a dose of 20 µl of the ImmunEasy™ per mouse. Tetanus toxoid (TT; Accurate Chemical & Scientific, Westbury, NY) contained 1058 Lf/ml and 2204 Lf/mg protein nitrogen. The various stock solutions were mixed together to form vaccine compositions for testing, as follows:

(i) TT (5 Lf/ml), 20% (v/w) ImmunEasy™ and 16.25% (w/w) Pluronic® F127;
(ii) TT (5 Lf/ml) and 20% (v/w) ImmunEasy™ (no Pluronic® F127);
(iii) TT (5 Lf/ml), 20% (v/w) ImmunEasy™ formulated with glycerol (no Pluronic® F127); and
(iv) TT (5 Lf/ml) and 20% (v/w) ImmunEasy™ formulated with incomplete Freund's adjuvant (no Pluronic® F127).

For composition (iii) TT/ImmunEasy™ in glycerol was prepared by mixing glycerol (approximately 99%; Sigma-Aldrich) with premixed TT/ImmunEasy™ in PBS. For composition (iv), TT in incomplete Freund's adjuvant (IFA) was prepared by emulsification of equal volumes of IFA (Sigma-Aldrich) and a 2x TT/ ImmunEasy™ mixture in PBS.

Immunization studies in mice: Balb/c female mice (Harlan, Indianapolis, IN), 6 to 8 weeks of age, were used for these studies. Groups of mice (n=4) were immunized once s.c. with 0.5 Lf TT in the various formulations on day 0.

Antibody assays: The serum antibody responses to TT were measured by ELISA. Wells of 96 well Nunc Maxisorb microtiter plates (Nunc, Gaithersburg, MD) were coated with 1 μg/ml TT in PBS. Plates were washed with PBS/0.05% Tween 20 and blocked with 1% bovine serum albumin (BSA) (Fisher Scientific, Pittsburgh, PA). Samples were serially diluted in PBS/0.1% BSA/0.05% Tween 20 (PBST) and added to wells in triplicate. Following incubation, plates were washed and goat anti-mouse IgG γ chain specific horseradish peroxidase (HRP)-labeled conjugate (Southern Biotechnology Associates Inc., Birmingham, AL) was added in PBST. After further incubation, antibody binding was detected with substrate buffer containing TMB (Sigma-Aldrich). Absorbance was read with an EIA reader (Molecular Devices, Sunnyvale, CA). Antibody titer was defined as the reciprocal of the dilution of serum that would yield an optical density of 0.5.

Serum samples were periodically collected over a 28 week period and analyzed for IgG anti-TT antibodies by ELISA. The numerical IgG antibody titer data at various time points following administration is presented in Exhibit D. Figure 14 graphically summarizes the IgG antibody titer data through week 16. Data from a representative experiment indicate that at 4 and 8 weeks, the presence of the Pluronic® F127 polymer significantly enhanced the IgG antibody response to TT compared to antigen/ImmunEasy™ alone ($p = 0.0023$ and 0.029 respectively). Furthermore, the response to TT/F127/ImmunEasy™ was significantly higher than that elicited by TT/ImmunEasy™/IFA ($p = 0.017$ and 0.029 respectively). TT/ImmunEasy™ was also combined with glycerol to make a comparison with another matrix used as both a cryoprotectant and a sustained release vehicle. However, this formulation caused no increase in the anti-TT immune response compared to TT/ImmunEasy™ .

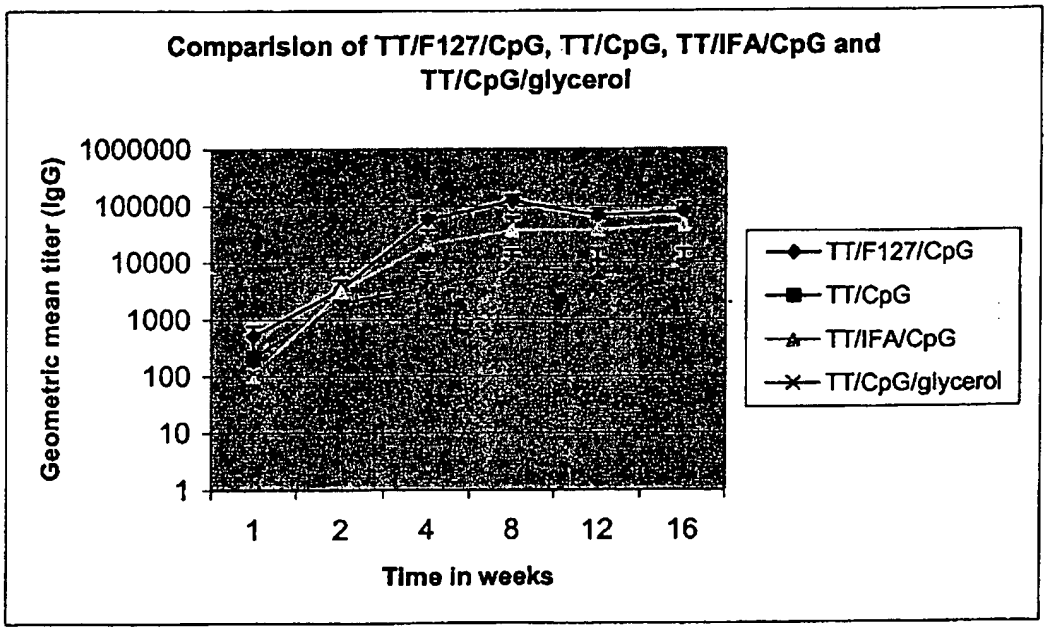

FIGURE 14

EXAMPLE 14: TT with CpG-containing adjuvant in the composition

Preparation of formulations: TT and Pluronic® F127 stock solutions were prepared as described in Example 1. ImmunEasy™ containing CpG as an adjuvant was added to formulations in an amount to provide a dose of 20μl, 6.7 μl or 2 μl of the ImmunEasy™ per mouse. Tetanus toxoid (TT; Accurate Chemical & Scientific, Westbury, NY) contained 1058 Lf/ml and 2204 Lf/mg protein nitrogen. The various stock solutions were mixed together to form vaccine compositions for testing, as follows:

(i) TT (5Lf/ml), 20% (v/w) ImmunEasy™ and 16.25% (w/w) Pluronic® F127;
(ii) TT (5 Lf/ml), 6.7% (v/w) ImmunEasy™ and 16.25% (w/w) Pluronic® F127;
(iii) TT (5 Lf/ml), 2% (v/w) ImmunEasy™ and 16.25% (w/w) Pluronic® F127;
(iv) TT (5 Lf/ml) and 20% (v/w) ImmunEasy™ (no Pluronic® F127);
(v) TT (5 Lf/ml) and 6.7% (v/w) ImmunEasy™ (no Pluronic® F127);
(vi) TT (5 Lf/ml) and 2% (v/w) ImmunEasy™ (no Pluronic® F127; and
(vii) TT (5 Lf/ml) and 16.25% (w/w) Pluronic® F127 (no ImmunEasy™).

Immunization studies in mice: Balb/c female mice (Harlan, Indianapolis, IN), 6 to 8 weeks of age, were used for these studies. Groups of mice (n=8) were immunized once s.c. with 0.5 Lf TT in the various formulations on day 0.

Antibody assays: The serum antibody responses to TT were measured by ELISA. Wells of 96 well Nunc Maxisorb microtiter plates (Nunc, Gaithersburg, MD) were coated with 1 μg/ml TT in PBS. Plates were washed with PBS/0.05% Tween 20 and blocked with 1% bovine serum albumin (BSA) (Fisher Scientific, Pittsburgh, PA). Samples were serially diluted in PBS/0.1% BSA/0.05% Tween 20 (PBST) and added to wells in triplicate. Following incubation, plates were washed and goat anti-mouse IgG γ chain specific horseradish peroxidase (HRP) labeled conjugate (Southern Biotechnology Associates Inc., Birmingham, AL) was added in PBST. After further incubation, antibody binding was detected with substrate buffer containing TMB (Sigma-Aldrich). Absorbance was read with an EIA reader (Molecular Devices, Sunnyvale, CA). Antibody titer was defined as the reciprocal of the dilution of serum that would yield an optical density of 0.5.

Serum samples were collected at weeks 2, 4, and 8 and assayed for the presence of IgG anti-TT antibodies by ELISA. The numerical IgG antibody titer data at various time points following administration is presented in Exhibit E. Figure 15 graphically summarizes IgG antibody titer data for compositions (iii), (vi) and (vii). The data indicate, for example, that at week 4, the formulation of TT with F127/ImmunEasy™(2%) already elicits a significantly higher response than that elicited by either component mixed with antigen alone ($p = 0.001$ vs. TT/ImmunEasy™ and $p = 0.0003$ vs. TT/F127).

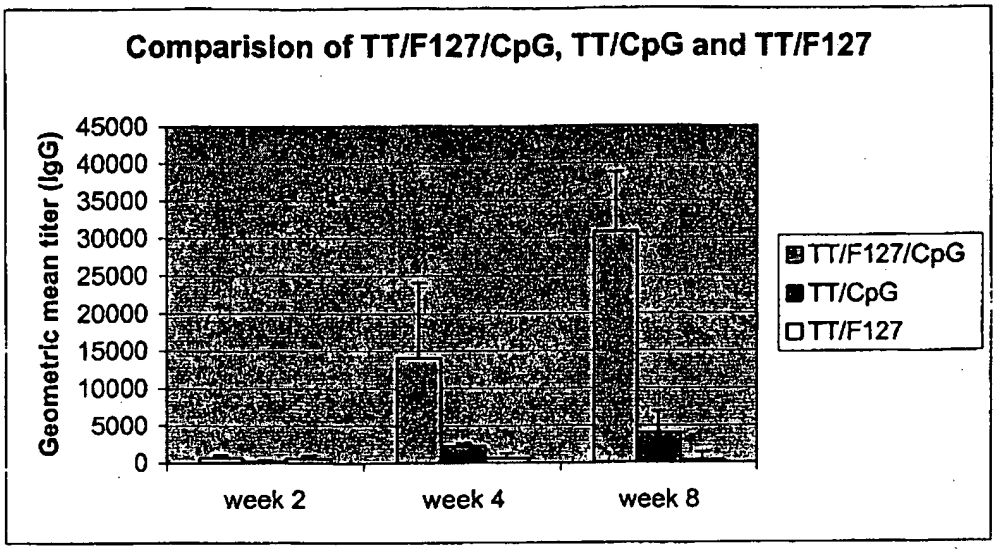

FIGURE 15

EXAMPLE 15: DT with CpG-containing adjuvant in the composition

Preparation of formulations: ImmunEasy™ containing CpG as an adjuvant was added to formulations in an amount to provide a dose of 20 µl of the ImmunEasy™ per mouse. Diphtheria toxoid (DT; Accurate) contained 2100 Lf/ml and 1667 Lf/mg protein nitrogen. The various stock solutions were mixed together to form vaccine compositions for testing, as follows:

(i) DT (1 Lf/dose), 20% (v/w) ImmunEasy™ and 16.25% (w/w) Pluronic® F127 and
(ii) DT (1 Lf/dose) and 20% (v/w) ImmunEasy™ (no Pluronic® F127).

Immunization studies in mice: Balb/c female mice (Harlan), 6 to 8 weeks of age, were used for these studies. Groups of mice (n=4) were immunized subcutaneously (s.c) with 1 Lf DT in the various formulations on day 0.

Antibody assays: The serum antibody responses to DT were measured by ELISA. Wells of 96 well Nunc Maxisorb microtiter plates (Nunc, Gaithersburg, MD) were coated with 10µg/ml DT in PBS. Plates were washed with PBS/0.05% Tween 20 and blocked with 1% bovine serum albumin (BSA) (Fisher Scientific, Pittsburgh, PA). Samples were serially diluted in PBS/0.1% BSA/0.05% Tween 20 (PBST) and added to wells in triplicate. Following incubation, plates were washed and goat anti-mouse IgG γ chain specific horseradish peroxidase (HRP)-labeled conjugate (Southern Biotechnology Associates Inc., Birmingham, AL) was added in PBST. After further incubation, antibody binding was detected with substrate buffer containing TMB (Sigma-Aldrich). Absorbance was read with an EIA reader (Molecular Devices, Sunnyvale, CA). Antibody titer was defined as the reciprocal of the dilution of serum that would yield an optical density of 0.5.

Serum samples were periodically collected over a 32 week period and analyzed for IgG anti-TT antibodies by ELISA. The numerical IgG antibody titer data at various time points following

administration is presented in Exhibit F. Figure 16 graphically summarizes IgG antibody titer data. Data from this experiment indicate, for example, that at 4 and 8 weeks after a single injection, the presence of the Pluronic® F127 polymer and ImmunEasy™ (composition (i)) antigen enhanced the IgG antibody response to DT compared to the use of ImmunEasy™ alone (composition (ii)).

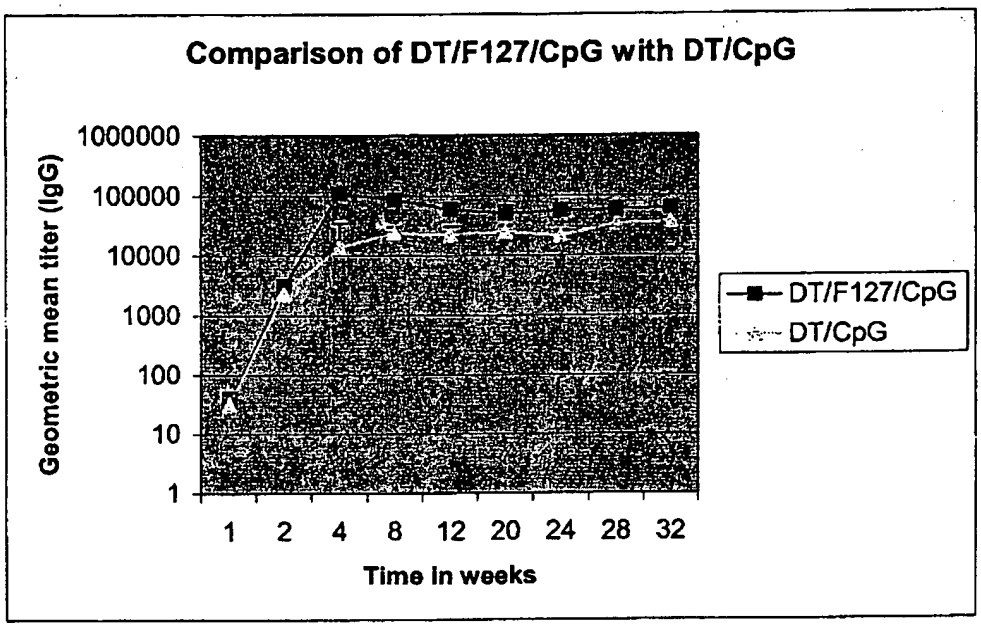

FIGURE 16

EXAMPLE 16: rPA with CpG-containing adjuvant in the composition

Preparation of formulations: ImmunEasy™ containing CpG as an adjuvant was added to formulations in an amount to provide a dose of 20 µl of the ImmunEasy™ per mouse. rPA was obtained from the NIH in the form of a lyophilized protein in 5 mM Hepes, pH 7.4. It was reconstituted in sterile water (USP grade) at 2 mg/ml before formulation. The various stock solutions were mixed together to form vaccine compositions for testing, as follows:

(i) rPA (250µg/ml), 20% (v/w) ImmunEasy™ and 16.25% (w/w) Pluronic® F127 and
(ii) rPA (250µg/ml) and 20% (v/w) ImmunEasy™ (no Pluronic® F127).

Also prepared was a third vaccine composition for testing, as follows:

(iii) rPA adsorbed to aluminum hydroxide (alum) was prepared by adsorption of rPA to Imject® alum (Pierce Endogen, Rockford, IL) according to manufacturer's instructions.

Immunization studies in mice: Balb/c female mice (Harlan, Indianapolis, IN), 6 to 8 weeks of age, were used for these studies. Groups of mice (n=6) were immunized s.c with 25µg rPA in the various formulations on day 0.

Antibody assays: The serum antibody response to rPA was measured by ELISA. The protective capacity of antibodies was measured in vitro using a toxin neutralization assay. For ELISA, wells of 96 well Nunc Maxisorb microtiter plates (Nunc, Gaithersburg, MD) were coated with 1 μg/ml rPA in PBS. Plates were washed with PBS/0.05% Tween 20 and blocked with 1% bovine serum albumin (BSA) (Fisher Scientific, Pittsburgh, PA). Samples were serially diluted in PBS/0.1% BSA/0.05% Tween 20 (PBST) and added to wells in triplicate. Following incubation, plates were washed and goat anti-mouse IgG γ chain specific horseradish peroxidase (HRP)-labeled conjugate (Southern Biotechnology Associates Inc., Birmingham, AL) was added in PBST. After further incubation, antibody binding was detected with substrate buffer containing TMB (Sigma-Aldrich). Absorbance was read with an EIA reader (Molecular Devices, Sunnyvale, CA). Antibody titer was defined as the reciprocal of the dilution of serum that would yield an optical density of 0.5.

Serum samples were periodically collected over an 12 week period and analyzed for IgG antibodies by ELISA. Figure 17 graphically summarizes IgG antibody titer data. The data indicate that rPA/F127/ ImmunEasy™ induced an early rise in IgG antibodies and that this response was significantly higher than the response to rPA/alum ($p < 0.05$).

Toxin Neutralization Assay (TNA): Serum samples were tested for their ability to prevent the lethal toxin (protective antigen + lethal factor (LF))-induced mortality of J774A.1 cells (American Type Culture Collection, Manassas, VA). Recombinant LF (rLF) was obtained from the NIH. Aliquots of 0.2 ml cell suspension (6 to $8x10^5$ cells/ml) in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) were plated into flat 96-well cell culture plates (Corning Costar, Acton, MA). Serial dilutions of pre- and post-immune serum samples were made in TSTA buffer (50 mM Tris pH 7.6, 142 mM sodium chloride, 0.05% sodium azide, 0.05% Tween 20, 2% BSA). PA and LF at final concentrations of 50 and 40 ng/ml respectively were added to each antiserum dilution. After incubation for 1 hour, 10 μl of each of the antiserum-toxin complex mixtures was added to 100 μl of J774A.1 cell suspension. The plates were incubated for 5 hours at 37°C in 5% CO_2. Twenty-five μl of 3-[4,5-dimethyl-thiazol-2-y-]-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) at 5 mg/ml in PBS was then added per well. After 2 hour incubation, cells were lysed and the reduced purple formazan solubilized by adding 20% (w/v) sodium dodecyl sulfate (SDS) in 50% dimethylformamide, pH 4.7. OD was read at 570 nm on an EIA reader. The lethal toxin-neutralizing antibody titers of individual serum samples, calculated by linear regression analysis, were expressed as the reciprocal of the antibody dilution preventing 50% of cell death and normalized to a control rabbit anti-rPA hyperimmune serum (NIH). Pre and post-immunization serum toxin neutralization titers were compared by the Sign test. Toxin neutralization titers between groups were compared by the use of the Mann Whitney U test. P values less than or equal to 0.05 were considered to indicate a significant difference.

The functional nature of the immune response to rPA was measured by TNA. The results of these studies (summarized graphically in Figure 18) indicate that formulation of rPA with F127/ ImmunEasy™ induces toxin neutralization titers significantly higher than formulation of rPA with alum ($p=0.002$) and rPA with ImmunEasy™ ($p=0.041$). The TNA titers were measured 8 weeks post immunization.

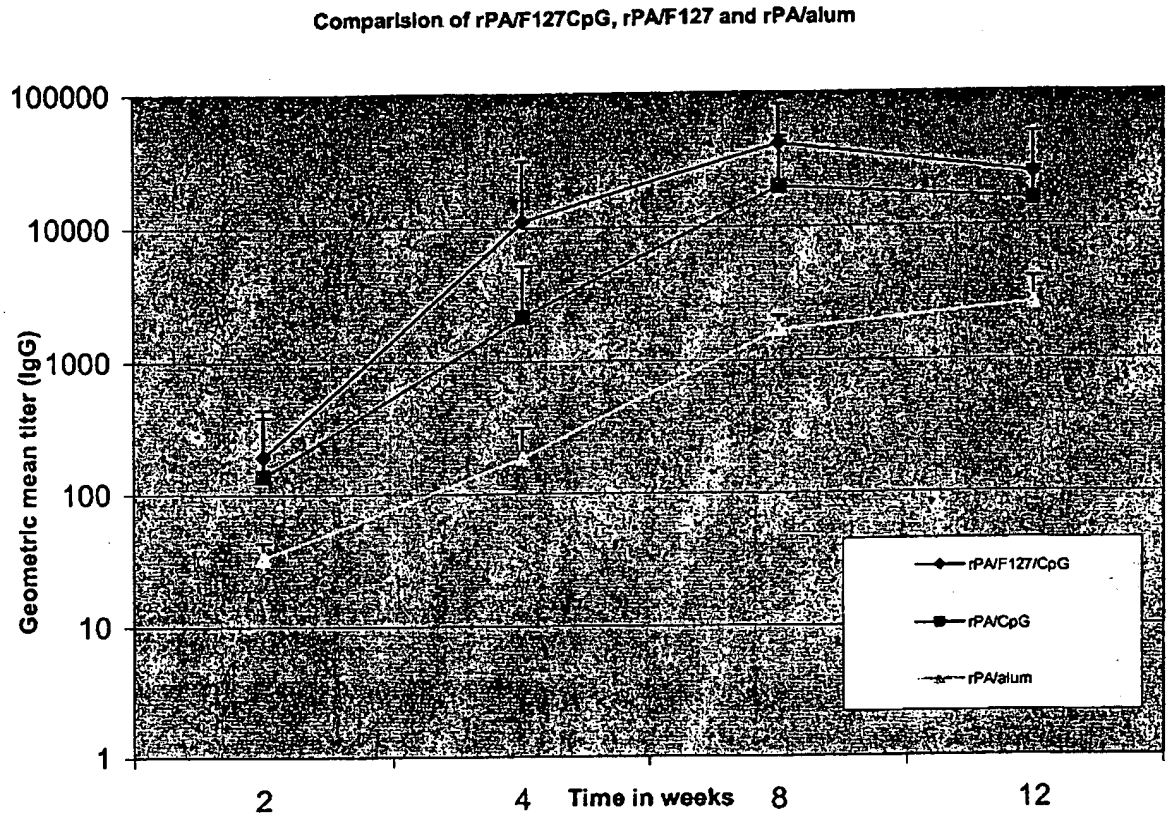

FIGURE 17

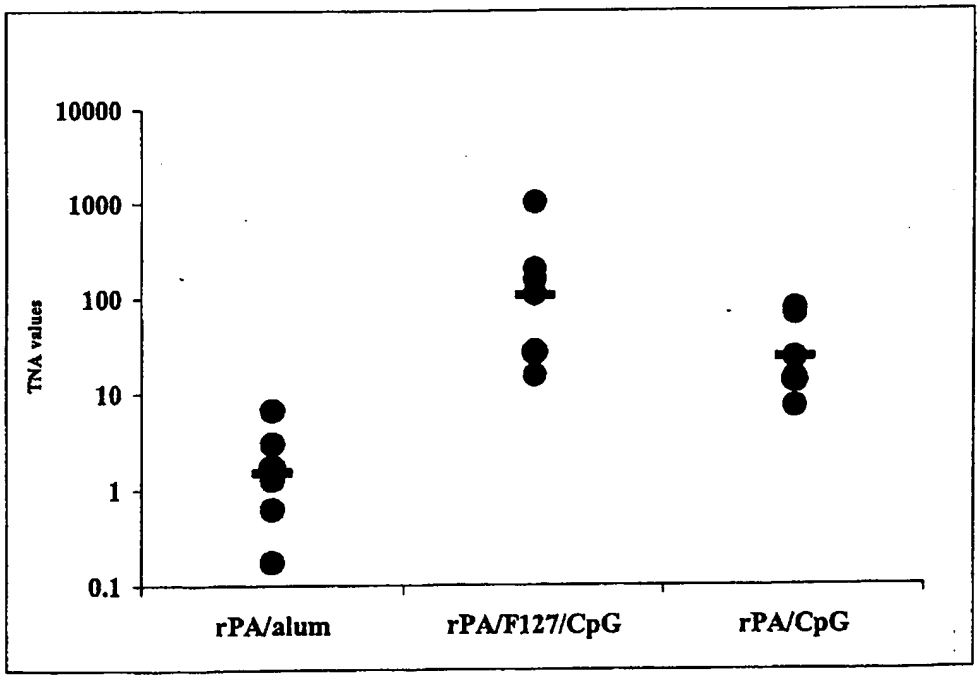

FIGURE 18

EXHIBIT C
TO RULE 132 DECLARATION OF
CLAIRE M. COESHOTT

EXAMPLE 12 – IgG ANTIBODY TITER DATA – CHITOSAN ADJUVANT

Formulation	**Animal**	**2 week**	**5 week**
0.5Lf TT/F127/Protasan® 0.5%	mouse 1-0	509	13187
	mouse 1-1	741	37839
	mouse 1-2	384	23253
	mouse 1-3	198	5989
	mouse 1-4	356	23257
	mouse 1-5	647	29727
	mouse 1-6	298	15262
	mouse 1-7	429	2170
	Geomean	**413**	**14132**
	Average	**445**	**18836**
	StDev	180	11990
0.5Lf TT/F127/Protasan® 0.17%	mouse 2-0	756	24180
	mouse 2-1	452	17380
	mouse 2-2	481	14339
	mouse 2-3	213	12748
	mouse 2-4	510	8873
	mouse 2-5	493	7991
	mouse 2-6	190	2610
	mouse 2-7	2227	17432
	Geomean	**497**	**11201**
	Average	**665**	**13194**
	StDev	656	6713
0.5Lf TT/F127/Protasan® 0.05%	mouse 3-0	279	2350
	mouse 3-1	184	3623
	mouse 3-2	357	3969
	mouse 3-3	359	6209
	mouse 3-4	92	5001
	mouse 3-5	298	7845
	mouse 3-6	290	23496
	mouse 3-7	310	3949
	Geomean	**252**	**5437**
	Average	**271**	**7055**
	StDev	91	6855

0.5Lf TT/Protasan® 0.5%	mouse 4-0	881	*29039
	mouse 4-1	291	5798
	mouse 4-2	13	4031
	mouse 4-3	698	9871
	mouse 4-4	8	3830
	mouse 4-5	DECEASED	DECEASED
	mouse 4-6	624	7050
	mouse 4-7	256	1840
	Geomean	**162**	**4748**
	Average	**396**	**5403**
	StDev	343	2824
0.5Lf TT/Protasan® 0.17%	mouse 5-0	171	4481
	mouse 5-1	620	8617
	mouse 5-2	409	11043
	mouse 5-3	1382	29896
	mouse 5-4	291	10183
	mouse 5-5	DECEASED	DECEASED
	mouse 5-6	299	DECEASED
	mouse 5-7	95	4431
	Geomean	**337**	**9119**
	Average	**467**	**11442**
	StDev	438	9465
0.5Lf TT/Protasan® 0.05%	mouse 6-0	377	14878
	mouse 6-1	292	9444
	mouse 6-2	179	6055
	mouse 6-3	345	8360
	mouse 6-4	144	3782
	mouse 6-5	176	2365
	mouse 6-6	273	2117
	mouse 6-7	98	2317
	Geomean	**215**	**4862**
	Average	**236**	**6165**
	StDev	100	4517
0.5Lf TT/F127	mouse 7-0	2	2
	mouse 7-1	25	193
	mouse 7-2	68	540
	mouse 7-3	85	252
	mouse 7-4	3	11
	mouse 7-5	163	431
	mouse 7-6	71	407
	mouse 7-7	31	472
	Geomean	**27**	**122**
	Average	**56**	**289**
	StDev	53	207

* outlier by Grubb's test not included in the analysis

EXHIBIT D

TO RULE 132 DECLARATION OF CLAIRE M. COESHOTT

EXAMPLE 13-IgG ANTIBODY TITER DATA-CpG ADJUVANT

Formulation	Animal	1 week	2 week	4 week	8 week	12 week	16 week	20 week	24 week	28 week
0.5Lf TT/F127/ImmunEasy™	mouse 1-0	922	6126	64626	84766	71894	76790	110056	113677	83564
	mouse 1-1	360	2762	71387	131447	42223	40959	114330	119421	127439
	mouse 1-2	345	2562	49756	129251	75097	102047	117018	109813	95376
	mouse 1-3	567	2936	46479	157242	87592	117944	33722	75090	69229
	Geomean	**505**	**3359**	**57152**	**122672**	**66857**	**78439**	**83943**	**102860**	**91572**
	Average	**549**	**3597**	**58062**	**125677**	**69202**	**84435**	**93782**	**104500**	**93902**
	StDev	269	1693	11886	30089	19219	33574	40142	20000	24783
0.5Lf TT/ImmunEasy™	mouse 2-0	50	1230	1762	6365	4520	2646	5554	7029	7165
	mouse 2-1	800	3118	6679	15961	11966	24294	36885	32372	31118
	mouse 2-2	384	1778	3553	5772	3251	4343	18529	32234	33193
	mouse 2-3	126	2233	4337	8919	10607	10262	22403	22312	16906
	Geomean	**210**	**1975**	**3670**	**8504**	**6572**	**7316**	**17077**	**20113**	**18807**
	Average	**340**	**2090**	**4083**	**9254**	**7586**	**10386**	**20843**	**23487**	**22096**
	StDev	338	799	2039	4675	4340	9830	12896	11940	12307
0.5LfTT/Glycerol/ImmunEasy™	mouse 3-0	118	1147	5779	10536	10957	10054	19997	22691	DECEASED
	mouse 3-1	355	1230	934	3138	1003	1745	2145	1432	DECEASED
	mouse 3-2	97	3602	7924	22344	25604	10425	38030	33281	DECEASED
	mouse 3-3	41	879	3993	9092	10536	10754	17639	18127	DECEASED
	Geomean	**114**	**1451**	**3615**	**9053**	**7379**	**6660**	**13024**	**11833**	
	Average	**153**	**1715**	**4658**	**11278**	**12025**	**8245**	**19453**	**18883**	
	StDev	139	1267	2957	8042	10153	4342	14700	13253	
0.5Lf TT/IFA/ImmunEasy™	mouse 4-0	124	3183	24224	34567	45628	46673	100221	104943	73511
	mouse 4-1	138	5819	29907	48098	51500	63457	83854	123394	126116
	mouse 4-2	42	1830	6884	15554	13082	14419	19158	20535	21779
	mouse 4-3	159	2969	43521	74077	64470	112450	179950	214709	220781
	Geomean	**103**	**3167**	**21584**	**37203**	**37520**	**46812**	**73366**	**86926**	**81711**
	Average	**116**	**3450**	**26134**	**43074**	**43670**	**59250**	**95796**	**115895**	**110547**
	StDev	51	1687	15174	24605	21859	40890	66125	79653	84942

EXHIBIT E
TO RULE 132 DECLARATION OF
CLAIRE M. COESHOTT

EXAMPLE 14 – IgG ANTIBODY TITER DATA – CpG ADJUVANT

Formulation	**Animal**	**2 week**	**4 week**	**8 week**
0.5Lf TT/F127/ImmunEasy™ 20ul	mouse 1-0	2452	4533	6527
	mouse 1-1	8540	10716	21931
	mouse 1-2	6134	7787	16381
	mouse 1-3	10410	5370	18023
	mouse 1-4	7820	18833	178204
	mouse 1-5	5482	DECEASED	DECEASED
	mouse 1-6	13655	25767	191955
	mouse 1-7	7148	155443	111427
	Geomean	**6974**	**14768**	**39903**
	Average	**7705**	**32636**	**77778**
	StDev	3354	54697	81435
0.5Lf TT/F127/ImmunEasy™ 6.7ul	mouse 2-0	1064	43386	227895
	mouse 2-1	3383	100388	101047
	mouse 2-2	1545	129383	173963
	mouse 2-3	2524	107859	146576
	mouse 2-4	1343	13211	658
	mouse 2-5	2197	100320	212735
	mouse 2-6	2930	254421	483347
	mouse 2-7	762	64369	30440
	Geomean	**1761**	**77632**	**76792**
	Average	**1969**	**101667**	**172083**
	StDev	936	72463	149667
0.5Lf TT/F127/ImmunEasy™ 2ul	mouse 3-0	512	19241	42222
	mouse 3-1	201	2002	*273
	mouse 3-2	701	27112	24730
	mouse 3-3	933	27112	23548
	mouse 3-4	708	29535	23802
	mouse 3-5	662	11502	31447
	mouse 3-6	1366	7779	40780
	mouse 3-7	1254	20145	35107
	Geomean	**694**	**14037**	**17065**
	Average	**792**	**18054**	**27739**
	StDev	382	10056	13333

* considered a non-responder removed from plotted data

Formulation	Animal	2 week	4 week	8 week
0.5Lf TT/ImmunEasy™ 20ul	mouse 4-0	3618	2081	2355
	mouse 4-1	2621	1556	2188
	mouse 4-2	5112	7978	12315
	mouse 4-3	10325	20929	131509
	mouse 4-4	5004	5947	1266
	mouse 4-5	9023	7291	19788
	mouse 4-6	4204	15859	658836
	mouse 4-7	6426	4827	136475
	Geomean	**5287**	**6050**	**14429**
	Average	**5792**	**8309**	**46467**
	StDev	2667	6756	57986
0.5Lf TT/ImmunEasy™ 6.7ul	mouse 5-0	523	3678	11148
	mouse 5-1	271	1952	2829
	mouse 5-2	170	2252	6186
	mouse 5-3	368	4555	19027
	mouse 5-4	1082	2005	1479
	mouse 5-5	674	5909	18458
	mouse 5-6	886	3116	17159
	mouse 5-7	458	4309	16667
	Geomean	**476**	**3225**	**8566**
	Average	**554**	**3472**	**11619**
	StDev	311	1411	7247
0.5Lf TT/ImmunEasy™ 2ul	mouse 6-0	254	2217	3005
	mouse 6-1	216	2440	4822
	mouse 6-2	248	1870	2360
	mouse 6-3	253	2122	6307
	mouse 6-4	373	3133	6657
	mouse 6-5	361	1757	2545
	mouse 6-6	456	1978	1897
	mouse 6-7	113	2741	10116
	Geomean	**264**	**2243**	**4034**
	Average	**284**	**2282**	**4714**
	StDev	107	467	2844

0.5Lf TT/F127	mouse 7-0	402	375	553
	mouse 7-1	880	763	965
	mouse 7-2	1025	727	273
	mouse 7-3	100	64	2
	mouse 7-4	1074	1548	1347
	mouse 7-5	1069	851	222
	mouse 7-6	1261	2104	*3364
	mouse 7-7	431	641	682
	Geomean	**623**	**626**	**345**
	Average	**780**	**884**	**926**
	StDev	414	650	1076

* outlier by Grubb's

EXHIBIT F
TO RULE 132 DECLARATION OF
CLAIR M. COESHOTT

EXAMPLE 15 – IgG ANTIBODY TITER DATA – CpG ADJUVANT

Formulation	Animal	1 week	2 week	4 week	8 week	12 week	16 week	20 week	24 week	28 week	32 week
1 Lf DT/ImmunEasy™	mouse 5-0	29	2497	50487	30118	33306	27301	28398	23309	28687	25079
	mouse 5-1	42	3391	6652	22451	24894	25266	26298	9622	32729	49216
	mouse 5-2	58	1325	14953	16828	14477	15250	18644	19928	20736	22238
	mouse 5-3	14	2567	7072	27854	20336	27631	28410	48629	66663	70600
	Geomean	**32**	**2317**	**13728**	**23727**	**22227**	**23219**	**25079**	**21592**	**33753**	**37310**
	Average	**36**	**2445**	**19791**	**24313**	**23253**	**23862**	**25438**	**25372**	**37204**	**41783**
	StDev	19	850	20817	5937	7943	5836	4637	16561	20262	22706
1 Lf DT/F127/ImmunEasy™	mouse 6-0	*9	1540	14847	6070	2928	2470	2880	3532	2250	2161
	mouse 6-1	19	2950	105873	69766	49986	37955	46485	49058	49500	47881
	mouse 6-2	37	2161	129855	98378	64515	54542	55037	57870	57101	59940
	mouse 6-3	44	4051	96437	87278	63688	18668	47777	60742	72827	76451
	Geomean	**23**	**2511**	**66609**	**43668**	**27847**	**17577**	**24358**	**27936**	**26087**	**26241**
	Average	**27**	**2676**	**86753**	**65373**	**45279**	**28409**	**38045**	**42801**	**45420**	**46608**
	StDev	16	1083	49959	41252	29010	22670	23743	26647	30375	31862

*mouse 6-0 omitted from graphic as low responder

APPENDIX C
RELATED PROCEEDINGS

None.